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Research Article

TCR Gene Transfer: MAGE-C2/HLA-A2 and MAGE-A3/HLA-DP4 Epitopes as Melanoma-Specific Immune Targets

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Adoptive therapy with TCR gene-engineered T cells provides an attractive and feasible treatment option for cancer patients. Further development of TCR gene therapy requires the implementation of T-cell target epitopes that prevent "on-target" reactivity towards healthy tissues and at the same time direct a clinically effective response towards tumor tissues. Candidate epitopes that meet these criteria are MAGE-$C2_{336-344}$/HLA-A2 (MC2/A2) and MAGE-$A3_{243-258}$/HLA-DP4 (MA3/DP4). We molecularly characterized TCR$\alpha\beta$ genes of an MC2/A2-specific CD8 and MA3/DP4-specific CD4 T-cell clone derived from melanoma patients who responded clinically to MAGE vaccination. We identified MC2/A2 and MA3/DP4-specific TCR-Vα3/Vβ28 and TCR-Vα38/Vβ2 chains and validated these TCRs *in vitro* upon gene transfer into primary human T cells. The MC2 and MA3 TCR were surface-expressed and mediated CD8 T-cell functions towards melanoma cell lines and CD4 T-cell functions towards dendritic cells, respectively. We intend to start testing these MAGE-specific TCRs in phase I clinical trial.

1. Introduction

Adoptive therapy with antigen-specific T cells has shown clinical successes in the treatment of viral infections and tumors [1–5]. Receptor gene therapy, in which patients are treated with gene-engineered T cells with either chimeric antigen receptors (CARs) or T-cell receptors (TCRs), provides an attractive alternative to provide therapeutic immunity. Clinical application of gene-engineered T cells to treat various tumor types, such as renal cell cancer, ovarian cancer, neuroblastoma, lymphoma, melanoma, and colorectal and synovial cancers proved feasible but, despite some successes, generally did not show antitumour responses in a substantial number of patients [6–13]. Notably, in an early clinical trial to treat metastatic renal cell cancer with CAR-engineered T cells, with total T-cell doses as low as 2×10^8 T cells, we observed reversible yet discrete cholangitis and damage to bile duct epithelium as a likely consequence of T-cell localization and expression of the target epitope carbonic anhydrase IX (CAIX) on normal tissue [6]. Subsequent trials with CARs directed against Her2/Neu and CD19 and TCRs directed against the HLA-A2-restricted antigens MARTI, gp100 and CEA, have confirmed this notion [11, 12, 14, 15]. Collectively, these studies underscore the need for T-cell target epitopes that are expressed on malignant tissue in a highly restricted manner and are able to initiate a clinically effective T-cell response.

Cancer testis antigens (CTAs) are immunogenic proteins expressed in many tumors but silenced in normal cells except for male germline cells, placenta, and thymic

medullary epithelial cells [16, 17]. *In vitro* studies have provided initial proof that gene transfer of TCR$\alpha\beta$ directed against MAGE-A1/HLA-A1, MAGE-A3/HLA-A2, and NY-ESO-1/HLA-A2 as well as NY-ESO-1/HLA-DP4 result in effective and CTA-specific T-cell responses [18–21]. Of the group of CTA, in particular the MAGE antigens constitute attractive candidates for immune therapy giving not only tumour-specific expression but also their role in tumour biology, expression in multiple tumours, and potential to constitute effective T-cell targets. Four families of MAGE genes are located on chromosome X: *MAGE-A (12 genes), B (6 genes), C (4 genes), and D (2 genes)*. Up to now, there are over 50 identified combinations of MAGE peptides and HLA class I or class II molecules, recognized by CD8 or CD4 T cells, respectively (see for an overview: http://cancerimmunity.org/peptidedatabase/Tcellepitopes.htm).

We propose the MAGE-C2$_{336–344}$/HLA-A2 (MC2/A2) peptide ALKVDVEERV and MAGE-A3$_{243–258}$/HLA-DP4 (MA3/DP4) peptide KKLLTQHFVQENYLEY as candidate T-cell targets for the following reasons. *First*, MC2 and MA3 proteins actively contribute to the development of malignancies. MC2 suppresses p53-dependent apoptosis, thus prolonging tumor survival [22, 23], whereas MA3 mediates fibronectin-controlled progression and metastasis [24], and is expressed by melanoma stem cells [25, 26]. *Second*, MC2 and MA3 are expressed in multiple tumor types and their expression is associated with poor clinical outcome in these tumor types [27–32]. MC2 is expressed in 43% of metastatic melanomas, 33% of head and neck squamous cell cancers, 30% of bladder cancers, and 10% of nonsmall cell lung cancers [28]. MA3 is expressed in 76% of metastatic melanomas [27], in up to 50% of nonsmall cell lung cancer [29], and in many other tumor types such as colon rectal, hepatocyte cellular, prostate and breast cancers, and haematological malignancies such as multiple myeloma [30, 33–36]. Furthermore, HLA-A2 and HLA-DP4 are the most frequent MHC class I and II alleles among Caucasians, that is, representing 44 and 75% of the general population, respectively. And *third*, MC2 and MA3 potentially constitute clinically effective T-cell target epitopes, as evidenced by induction of enhanced numbers of anti-MAGE T cells that paralleled significant and durable clinical responses [37, 38].

The clinical potential of MC2-specific T cells is exemplified by a high frequency of MC2/A2-specific CTL (10^{-4} of CD8 T cells) observed in the blood of a melanoma patient whose tumors regressed after vaccination with MAGE-A1 and A3 peptides, whereas in the same patient the frequency of anti-vaccine CTLs was low (3×10^{-6} of CD8 T cells) [37]. A CTL clone recognizing this epitope (EB81-CTL16) was isolated demonstrating the most pronounced increase in frequency not only in blood but also in a regressing cutaneous metastasis (>100 and 1000-fold, resp.). Interestingly, the same patient also showed increased frequencies in blood and a regressing metastasis (up to 200-fold) of other T-cell clones-specific for the same and other MC2 epitopes [39]. In a second melanoma patient who showed tumor regression upon MAGE vaccination, the most frequent antitumor CTL clone was again directed against a MC2 epitope [40]. With respect to MA3, various trials have been performed substantiating its clinical potential as a T-cell target. A phase II clinical trial with highly purified MA3 protein in nonsmall cell lung cancer showed a significant reduction in relative risk of cancer recurrence following surgery in vaccinated versus placebo-treated patients [41]. This MA3 vaccine provided B-cell responses, CD8 T-cell responses as well as HLA-DP4-restricted CD4 T-cell responses against the MA3 KKL epitope in lung cancer patients [42, 43]. Recently, a phase III trial started to investigate the efficacy of MA3 antigen vaccination after tumor resection in lung cancer patients [44]. Also in melanoma patients, MA3 protein vaccinations using either protein or MA3-expressing PBMC initiate antigen-specific immune responses [45]. Vaccinations with dendritic cells loaded with MA3/DP4 peptide rapidly induced peptide-specific T-helper-cell responses in melanoma patients. Median survival in vaccinated patients was longer than in untreated control patients and showed no signs of major toxicities due to vaccination [46] and personal communication (Gerold Schuler, Erlangen, Germany).

In this study, we chose MC2$_{336–344}$/A2 and MA3$_{243–258}$/DP4 as T-cell epitopes, and cloned and characterized the corresponding TCR$\alpha\beta$ genes of CD8 and CD4 T-cell clones derived from two metastatic melanoma patients who responded clinically to MAGE-vaccination. TCR$\alpha\beta$ genes were then introduced into primary human T cells, and tested for surface expression and MAGE-specific CD8 and CD4 T-cell functions *in vitro*.

2. Materials and Methods

2.1. Melanoma Patients EB81 and R12 and Patient-Derived T-Cell Clones. In a vaccination study, metastatic melanoma patient EB81 received cutaneous vaccinations with recombinant canarypox (ALVAC) virus, carrying a minigene encoding antigenic MAGE-A1 and A3 peptides that are presented by HLA-A1. These were followed by vaccinations with the same peptides. One year after the first vaccination, all cutaneous metastases had disappeared, and the patient remained tumor-free for 3 years [37]. CTL-606C/22.2 (EB81-CTL 16) is a cytotoxic CD8 T-cell clone derived from EB81 whose increase in frequency is most pronounced in various body compartments upon vaccination with MAGE, and it recognizes MC2$_{336–344}$/HLA-A2 (ALKVDVEERV) [37]. Melanoma patient 12 was included in a clinical trial in which mature monocyte-derived dendritic cells loaded with multi-HLA class I and II peptides (including MAGE-A3$_{243–258}$ peptide) were administered subcutaneously [38]. CD4 T-cell clone R12-C9, recognizing MA3$_{243–258}$/HLA-DP4 (KKLLTQHFVQENYLEY), was derived from PBMC from melanoma patient 12, after *in vitro* stimulation with MA3$_{243–258}$/DP4 peptide and sorted on IFNγ secreting CD4+ T cells by FACSVantage flow cytometer (BD Biosciences) as described earlier [46]. CTL clones 16 and R12-C9 were cultured in IMDM with 10% human serum, glutamine, and antibiotics.

2.2. Other Cells and General Reagents. PBMC from healthy donors were isolated by centrifugation through Ficoll-Isopaque (density = 1.077 g/cm^3; Amersham Pharmacia Biotech, Uppsala, Sweden). Transduced primary human T cells were cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 200 mM L-glutamine, 10% human serum, antibiotics, and 360 IU/mL human rIL-2 (Proleukin; Chiron, Amsterdam, The Netherlands) and stimulated every 2 weeks with a mixture of irradiated allogeneic feeder cells as described elsewhere [47]. The human embryonic kidney cell line 293T and Phoenix-Ampho, both used to package retroviruses carrying RNA encoding TCR$\alpha\beta$, were grown in DMEM with 10% fetal bovine serum (FBS; Greiner Bio-one Alphen a/d Rijn, The Netherlands), glutamine, antibiotics, and 1% MEM nonessential amino acids. The same medium plus supplements was used to grow the melanoma cell lines EB-81-MEL-2 (MC2/A2^{pos}) and MZ2-MEL43 (MA3/DP4^{pos}). An MC2^{neg}/A2^{pos} and MA3^{neg}/DP4^{pos} B lymphoblast cell line (BSM) and an EBV transformed HLA-DP4^{pos} B cell line (i.e., EBV-MAGJ) transduced with retrovirus encoding li-MA3 cDNA as described in [48] (i.e., EBV-MA3) were cultured in RPMI supplemented with glutamine, antibiotics, and 10% FBS. The melanoma cell line EB-81-MEL-2 and the B cell line EBV-MAGJ were derived from the same patients from whom the T-cell clones were derived (melanoma patient EB-81 and patient 12, resp.). In some cases, target cells were pretreated with 50 pg/mL human recombinant IFNγ (Peprotech, Rocky Hill, NJ, USA) for 48 h prior to functional T-cell assays.

MC2/A2 peptide MHC (pMHC) complexes were ordered from Proimmune (Oxford, UK). MA3/DP4 pMHC complexes were produced in S2-drosophila insect cells, essentially as described previously [46]. We used the following mAbs: anti-CD4 (clone 13 B8.2, BD Biosciences, Erembodegem, Belgium), anti-CD8 (clone SK1, BD Biosciences) and anti-TCR-Vβ2 mAbs (clone MPB 2D5, Immunotech, Marseille, France). Other reagents used were the HLA-A2-binding peptides MC2$_{336–344}$ (ALKVDVEERV) and (as a control) gp100$_{280–288}$ (YLEPGPVTA), the HLA-DP4-binding peptide MA3$_{243–258}$ (KKLLTQHFVQENYLEY) (all three from Eurogentec, Maastricht, The Netherlands), Phytohemagglutinin (PHA) (Remel Ltd, Lenexa, KS, USA), Phorbol 12-Myristate 13-Acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA), GM-CSF, IL-4, TNFα (all three from PeproTech) and PGE2 (Sigma-Aldrich).

2.3. MAGE-A3 Protein. MA3 protein was expressed by the Des insect cell expression system (Invitrogen, Breda, The Netherlands). To this end, MA3 cDNA was cloned into the pMT/BiP/V5-His vector and, together with the pCoHygro vector, introduced into S2-insect cells by nucleofection (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's guidelines. MA3 protein expression by transfected S2 cells, at a density of 3×10^6/mL, was induced by copper sulfate (500 μM). Five days after induction of protein expression, culture medium was harvested and soluble MA3 protein was purified by FPLC (Acta, GE Healthcare, Zeist, The Netherlands) using a histrap column, followed by size exclusion on a sephadex 75 column.

2.4. Genes Encoding TCR$\alpha\beta$-Specific for MC2/A2 and MA3/DP4. RNA was isolated from T-cell clones EB81-CTL16 and R12-C9 and reverseLy transcribed with Superscript III (Invitrogen) according to the manufacturer's instructions. The TCR-Vα and Vβ regions were amplified and family-typed using a set of sense primers, covering all variable segments, in combination with either a TCR-Cα or Cβ antisense consensus primer. Nested PCR was performed on TCR-Vα and Vβ products before gel electrophoresis. Primers specific for Cβ1 or Cβ2 were used to discriminate between both Cβ genes. Positive PCR products were cloned, and plasmid DNAs from at least 5 independent colonies were sequenced. Specific primers were then used to amplify full-length (FL) TCRα and β DNAs from CTL-derived cDNA. In some cases, (i.e., MA3/DP4 TCRβ) primers were also used to amplify control TCR DNAs from a spleen cDNA library. Standard primers were used to amplify the TCRα and TCRβ DNAs and will be provided upon request. TCRα and β genes were cloned as wild-type TCRs into two separate pBullet retroviral vectors [49] (abbreviated as pB: TCRα+β) or as codon-optimized TCRs in a TCRβ-2A-TCRα cassette in a single pMP71 vector (abbreviated as pMP71: optTCRβ-2A-TCRα, see *Supplementary text and figures available online at doi: 10.1155/2012/586314*). The strategy we employed to clone MAGE-specific TCR$\alpha\beta$ genes and to test their surface expression and function following TCR gene transfer is depicted in Figure 1(a).

2.5. Transduction of Human T Lymphocytes. Human T lymphocytes of healthy donors were activated with anti-CD3 mAb and transduced with retrovirus harboring either MAGE-specific or control TCRα and β transgenes. The transduction procedure was described by Lamers and colleagues [50] except that in the current study TCR-encoding retroviruses were produced by a coculture of 293T and Phoenix-Ampho packaging cells. T cells were FACSorted using the corresponding p/MHC multimer prior to functional assays. For some experiments, the MA3/DP4 TCR transduced T cells were depleted either for CD4 or CD8 T cells using anti-CD4 or CD8 mAb-coated and PE-labeled magnetic beads and MACS columns (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

2.6. Flow Cytometry of TCR-Transduced T Lymphocytes. MC2/A2 TCR-transduced T cells were analyzed for TCR expression by flow cytometry using PE-labeled MC2/A2 pentamers (10 nM). MA3/DP4 TCR-transduced T cells were analyzed for TCR expression by PE-labeled anti-TCR-Vβ2 mAb, PE-labeled MA3/DP4 tetramers (50 nM), and anti-CD4 mAb. For immunostaining, 0.5×10^6 transduced T cells were washed with PBS and incubated with MC2/A2 pentamer (or antibodies) at 4°C for 30 min or with MA3/DP4 tetramer at 37°C for 2 h. Upon completion of the immunostainings, cells were washed and fixed with 1% paraformaldehyde. Events were acquired and analyzed

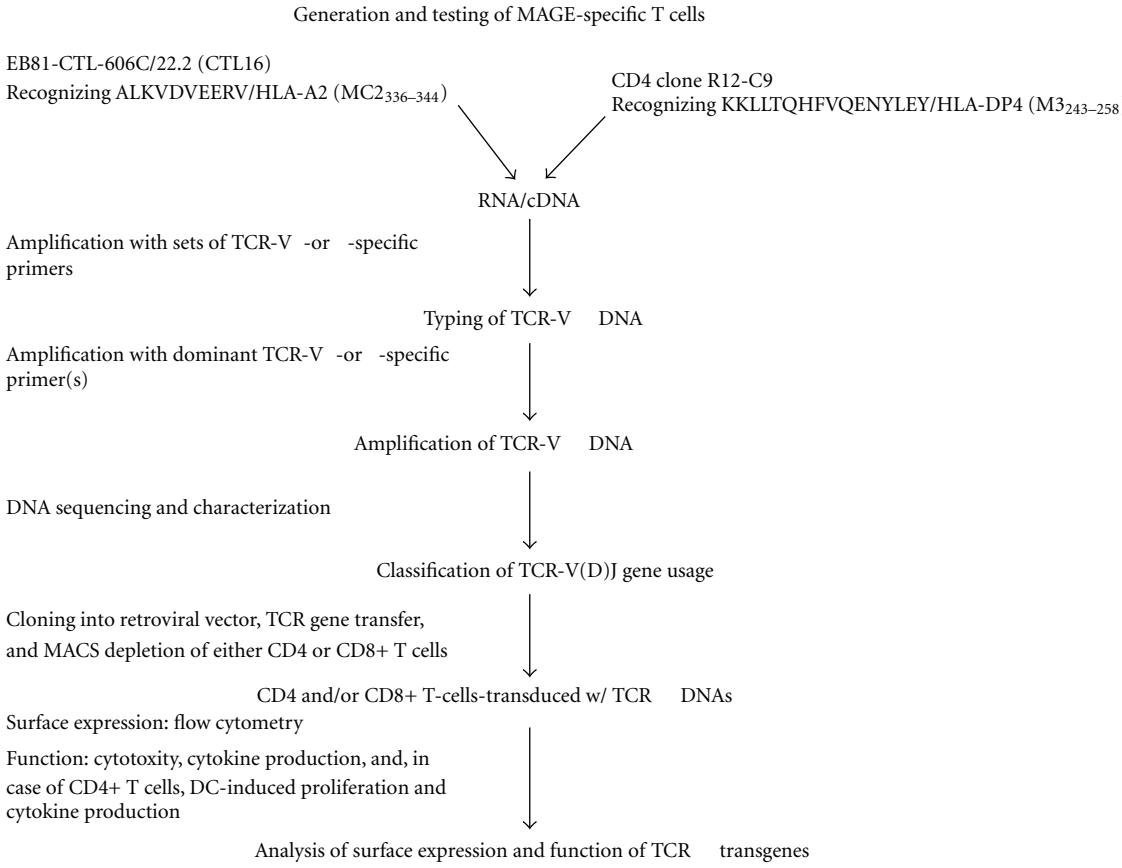

Figure 1: Cloning and validation of MC2/A2 and MA3/DP4 TCR$\alpha\beta$ genes. (a) Schematic representation of how TCR DNAs have been cloned, typed for TCR-V(D)J gene usage, and tested in T cells following gene transfer. (b) TCR-V(D)J and C classification of the TCRα and β chains expressed by EB81-CTL16 and R12-C9 according to http://www.imgt.org/. The arrow before the Jα6.01 indicates a frame shift preventing surface expression of this TCR-Vα2 chain. Sequence data for human TCR-Vα2, Vα3, and Vβ28 of EB81-CTL16-derived TCR genes are available from GenBank under accession nos. EU427373, EU427374, and EU427375, respectively; and sequence data for human TCR-Vα38 and Vβ22 of R12-C9-derived TCR genes are available from GenBank under accession nos. EU427376 and EU427377, respectively.

on a Cytomics FC 500 flow cytometer with CXP software (Beckman Coulter, Mijdrecht, The Netherlands).

2.7. Cytotoxicity Assay. Cytotoxic activity of T cells was measured in a standard 6 h ^{51}Cr-release assay as described previously [51]. Peptide loading of target cells was performed by addition of either MC2, gp100 (control) or MA3 peptide (final concentrations at 10 μM) for 15 min at 37°C and 5% CO_2 prior to cocultivation with effector T cells.

2.8. Cytokine Production. To quantify the production of cytokines after antigen-specific stimulation, 6×10^6 T cells were cultured in the presence of 2×10^6 target cells for 18 h at 37°C and 5% CO_2. As a positive control, T-cell transductants were stimulated with PHA and PMA. Supernatants were harvested, and levels of IFN-γ and TNF-α were determined by standard ELISA (Sanquin, Amsterdam, The Netherlands).

2.9. CD4 T-Cell Assay. CD4 T-cell assays were based on dendritic cell: CD4 T-cell cocultures. To generate autologous dendritic cells (DCs), we used PBMC from the same HLA-DP4-positive donor that had been used to generate MA3/DP4 TCR-transduced CD4 T cells. PBMC were MACS-enriched using CD14 microbeads (Miltenyi Biotech), seeded at 10^6 cells/mL in RPMI 1640 medium without HEPES and supplemented with glutamine, 10% FBS, 10 μg/mL gentamycine, and the cytokines GM-CSF (1000 IU/mL) and IL-4 (200 IU/mL). At day 6, cells were used as a source of immature DC and incubated with MA3 protein (25 μg/mL) either in the absence or presence of TNFα (200 IU/mL) and PGE2 (5 μM) for an additional 2 days resulting in immature or mature MA3-positive DC, respectively. DC maturation state was confirmed by flow cytometric analysis of surface expression of CD80, CD86, and HLA-DR.

Immature or mature MA3-positive DCs were washed and added at 2×10^4 per round-bottomed microwell to 2×10^5 CD4 T cells in 200 μl T-cell medium. After 4 days of DC: T-cell coculture, supernatants were harvested, and cytokine production was determined in culture supernatants with Cytokine Bead Array (Th1/Th2 CBA kit, BD Biosciences) according to the manufacturer's instructions.

3. Results

3.1. Sequences of TCR$\alpha\beta$ Genes from MAGE-Specific T-Cell Clones. CD8 T-cell clone EB81-CTL16 and CD4 T-cell clone R12-C9, which were established from melanoma patients following MAGE vaccinations, were used to obtain genes encoding for MC2/A2- and MA3/DP4-specific TCR$\alpha\beta$'s. Sequence characterization revealed that EB81-CTL 16 harbored genes encoding TCR-Vα2.01/Jα6.01/Cα, Vα3.01/Jα35.01/Cα, and Vβ28.01/Jβ1-6.01/Cβ1, whereas R12-C9 harbored genes encoding TCR-Vα38.02/Jα52.01/Cα and Vβ2.01/Dβ1.01/Jβ1-2.01/Cβ2. We found that the TCR-Vα2.01/Jα6.01/Cα contained a frame shift in the Jα region (Figure 1(b)). As a result, there was a premature stop codon in the constant domain and no surface expression of this TCRα chain (see Figure 2(a)). Figure 1(b) shows the exact nucleotide and amino acid sequences of the various MAGE TCR chains and their corresponding TCR-V(D)J and C classifications (according to http://www.imgt.org/).

3.2. TCR-Vα3/Vβ28 Chains Confer T Cells with the Ability to Bind MC2$_{336–344}$/A2 Ligands. Retroviral transduction of human primary T cells with the TCR-Vα3Cα and Vβ28Cβ1 chains but not with irrelevant TCRα and β chains (i.e., mock TCR) resulted in TCR surface expression and binding to multimers of recombinant HLA-A2 molecules folded with MC2$_{336–344}$ peptide (Figure 2(a)). Enrichment of TCR-transduced T cells (TCR T cells) with MC2/A2 multimers by FACSort resulted in higher proportions of T cells expressing the MC2/A2 TCR (30% versus 65% pMHC binding before and after sort, Figure 2(b)). TCR surface expression was stable for at least three months (data not shown).

3.3. TCR-Vα3/Vβ28-Transduced Primary Human T Cells Show Antigen-Specific Functions In Vitro. To assess the antigen-specific cytolytic function of MC2/A2 TCR T cells, T cells were cocultured with the MC2/A2-positive tumor cell line, EB81-MEL-2. Figure 3(a) shows that if these tumor cells were pretreated with IFNγ, they were lysed by the TCR T cells. MC2 peptide-loaded HLA-A2 positive B cells (BSM) were lysed very efficiently, whereas gp100 peptide-loaded B cells were not recognized. Additionally, TCR T cells produced IFNγ but not TNFα in response to IFNγ pretreated EB81-MEL-2 cells, although T cells produced both IFNγ and TNFα in response to MC2 peptide-loaded cells (Figure 3(b)). No IFNγ was produced by MC2/A2 TCR T cells in response to MC2^{pos}/A2^{neg} tumor cells (*Supplementary Figure 1(b)*).

3.4. TCR-Vα38/Vβ2 Chains Provide T Cells with the Ability to Bind MA3$_{243–258}$/HLA-DP4 Ligands. MA3/DP4 TCR-transduced T cells, whether depleted or not for either the CD4 or CD8 T-cell subset, expressed high levels of TCR-Vβ2 (Figure 4(a)). Sorting the T cells after gene transfer for high-pMHC binding resulted in expressions that improved by a factor of two (21 and 26% pMHC binding prior to sorting versus 48 and 44% post sorting for CD4 and CD8-depleted T cells, resp.) (see Figure 4(b)). Mock T cells did not bind MA3/DP4 pMHC multimers. Similar to the MC2/A2 TCR, MA3/DP4 TCR expression was stable for at least three months (data not shown).

3.5. MA3/DP4 TCR T Cells Recognize Antigen-Positive B Cells but Not Melanoma Cells Natively Expressing MA3 Antigen. MA3/DP4 TCR T cells specifically lysed MA3/DP4 positive EBV B cells (EBV-MA3) (Figure 5(a)). Depleting MA3/DP4 TCR T cells for CD8 T cells resulted in CD4 T cells with a cytotoxic capacity similar to that of nondepleted T cells (mainly being of the CD8 T-cell subset). T cells transduced with the TCRβ chain of the MA3/DP4 TCR and a TCRα chain from a human spleen cDNA library served as a negative control (referred to as Mock T cells) and did not lyse MA3-positive B cells (Figure 5(a)). MA3/DP4 TCR T cells did not lyse MZ2-MEL43 melanoma cells, which naturally express

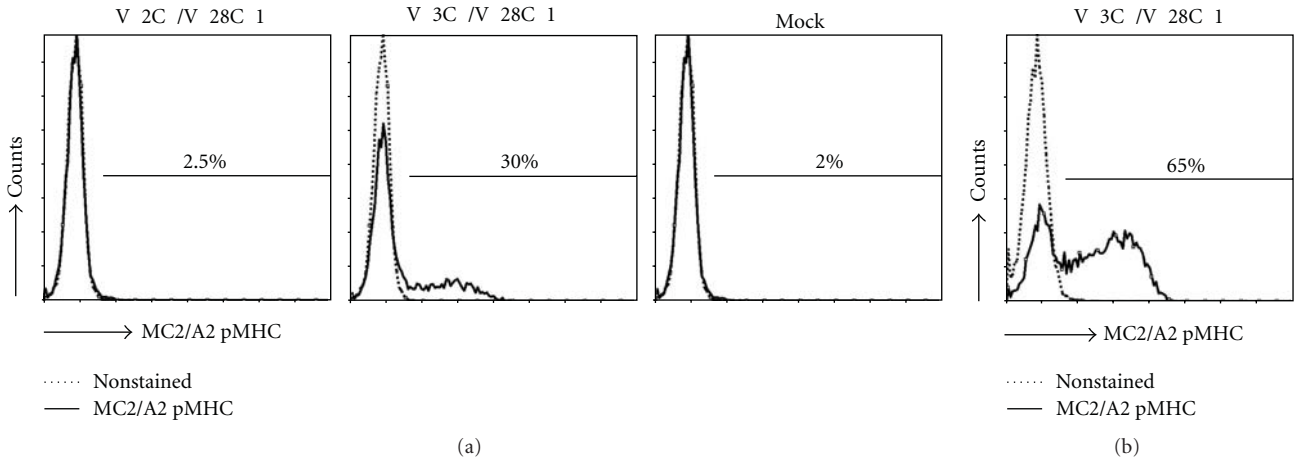

Figure 2: Primary human T cells transduced with TCR-Vα3/Vβ28 genes bind MC2/A2 pMHC. The MC2/A2 TCR T cells were labeled with PE-conjugated $MC2_{336–344}$/A2 pentamers before flow cytometric analysis (solid lines). (a) T cells transduced either with TCR-Vα2Cα/Vβ28Cβ1 and Vα3Cα/Vβ28Cβ1 or control TCR$\alpha\beta$ genes (Mock), and not sorted for MC2/A2 binding. (b) T cells transduced with TCR-Vα3Cα/Vβ28Cβ1 genes and FAC Sorted with MC2/A2 pentamer. Results are from a representative transduction out of 6 transductions of PBMC from 2 donors with similar results.

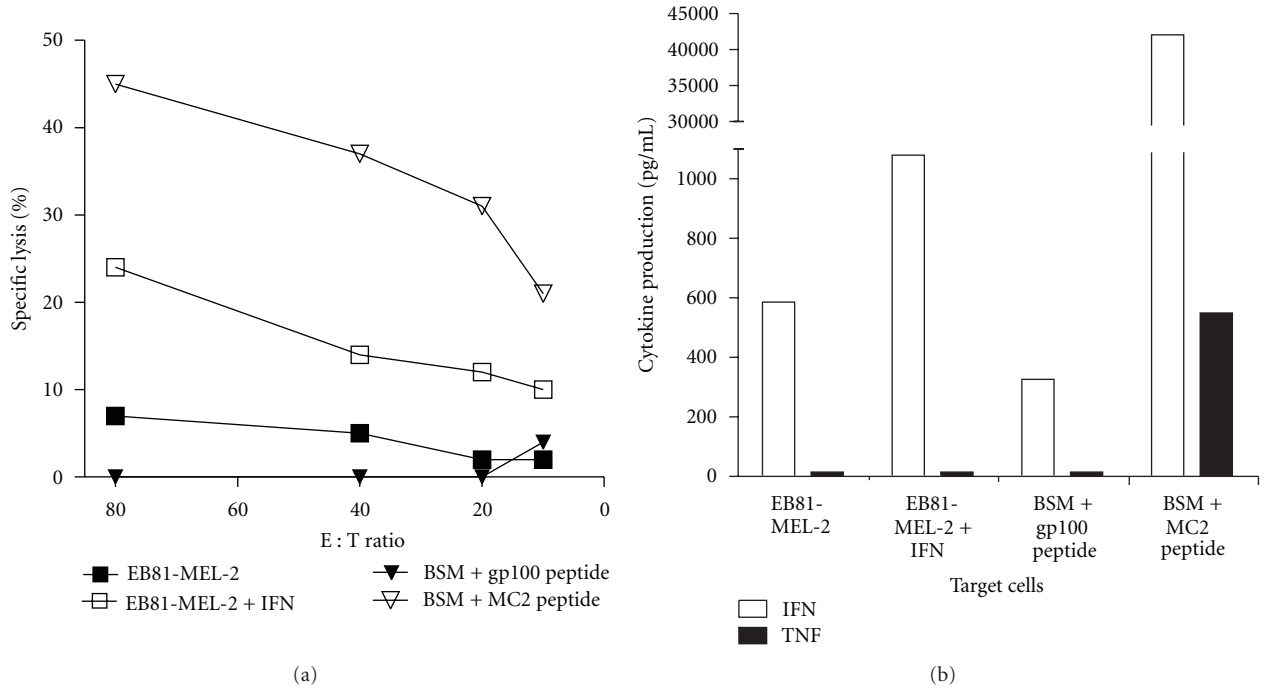

Figure 3: MC2/A2 TCR is functionally expressed by primary human T cells. (a) MC2/A2 TCR T cells lyse MC2/A2 positive target cells. TCR T cells were tested in a 6 h ^{51}Cr-release assay. The following target cells were used: MC2/A2-positive EB81-MEL-2 melanoma cells (derived from the same patient from whom the MC2 TCR was derived), pretreated or not with IFNγ, and A2-positive BSM EBV-B cells, pulsed either with gp100 or MC2 peptide (both at 10 μM final). Mock T cells did not lyse MC2/A2-positive target cells (data not shown). Effector-to-target cell ratios are indicated on the x-axis and specific ^{51}Cr-releases are indicated on the y-axis. (b) MC2/A2 TCR T cells produce cytokines upon coculture with MC2/A2-positive target cells. T-cell production of IFNγ and TNFα (in pg/mL) was measured by ELISA in supernatants harvested after an 18 h coculture between T cells and the target cells described in legend to Figure (a) No cytokines were produced by T cells only or Mock T cells cocultured with MC2-positive target cells (data not shown). Measurements were performed in triplicate and expressed as mean values corrected for medium values. Data shown are from representative experiments out of 4 experiments from 2 donors with similar results.

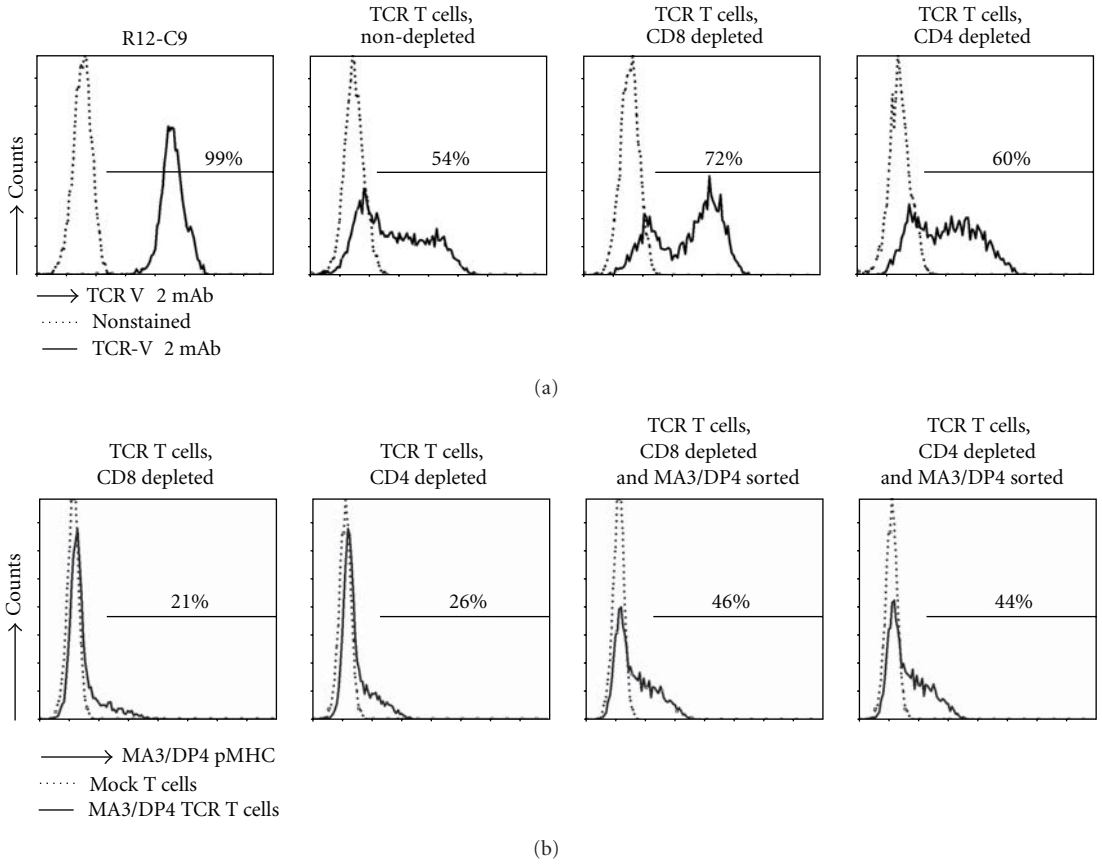

Figure 4: Surface expression of MA3/DP4 TCR on human primary T cells following gene transfer. Human primary T cells transduced with MA3/DP4 TCR$\alpha\beta$ genes were stained with TCR-Vβ2 mAb (in which case nonstained MA3/DP4 TCR T cells served as a negative control since control TCR$\alpha\beta$ genes also comprise the TCR-Vβ2 chain) (a) or MA3/DP4 tetramer (b) prior to analysis by flow cytometry. In (a), the following T cells were analyzed: parental CD4 T-cell clone R12-C9; TCR T cells, nondepleted (bulk) and TCR T cells depleted for either CD8 or CD4 T cells. These T-cell populations are not FAC sorted. In (b), TCR-transduced T cells, depleted for either CD8 or CD4 T cells nonsorted, or FAC sorted with MA3/DP4 tetramer, were analyzed. Results are from a representative transduction out of 4 transductions of PBMC from 2 donors with similar results.

the MA3/DP4 antigen (Figure 5(b)). Pretreatment with IFNγ did not, but addition of MA3 peptide did, enhance killing of the MZ2-MEL43 melanoma cells by MA3/DP4 TCR T cells (Figures 5(c) and 5(d)). Next, we showed that MA3/DP4 TCR T cells, but not Mock T cells, produced IFNγ and to a lesser extent TNFα in response to EBV-MA3 cells, with CD4 T cells as the predominant source of both cytokines (Figure 6(a)). It is noteworthy that TCR CD4 T cells, but not the parental R12-C9 T-cell clone, produced more IFNγ than TNFα (Figure 6(a)). Responses of TCR T cells towards EBV-MA3 B cells were blocked with an anti-TCR Vβ2 antibody, whereas those towards MA3^{pos}/DP4^{neg} tumor cells (*Supplementary Figure 2(b)*) and MA3^{neg}/DP4^{pos} B cells were always negative (data not shown). T cells expressing MA3/DP4 TCR (but not Mock) were able to respond to MZ2-MEL43 melanoma cells only when target cells were preloaded with MA3 16 mer peptide; this demonstrates that these melanoma cells can be sensitized to peptide-specific T-cell functions (i.e., cytotoxicity: data not shown; production of IFNγ and TNFα: Figure 6(b)).

3.6. MA3/DP4 TCR CD4 T Cells Produce Cytokines upon Coculture with MA3-Loaded Autologous Monocyte-Derived DC. Since MA3/DP4 TCR T cells are unable to directly recognize antigen-positive melanoma cells, an ability that is generally expected only for antitumor CD8 T cells, we analyzed a more typical CD4 T-cell response that is based on (cross-) presentation of tumor antigens by DC. To this end, MA3/DP4 TCR and Mock CD4 T cells were cocultured with immature or mature DC derived from autologous monocytes using two different MA3 protein concentrations for DC uptake. After 4 days, the production of various cytokines was determined in supernatants of the DC: T-cell cocultures. Upon coculture with the MA3-protein-loaded DC, MA3/DP4 TCR CD4 T cells (but not Mock CD4 T cells) produced significant amounts of IFNγ (up to 1300 pg/mL) and to a lesser extent TNFα, IL-2, IL-4, and IL-5 (Figure 7). MA3-specific production of IL-10 was negligible. T-cell cytokine production was negligible or absent when either nonprotein-loaded immature or mature DC or Mock T cells were used.

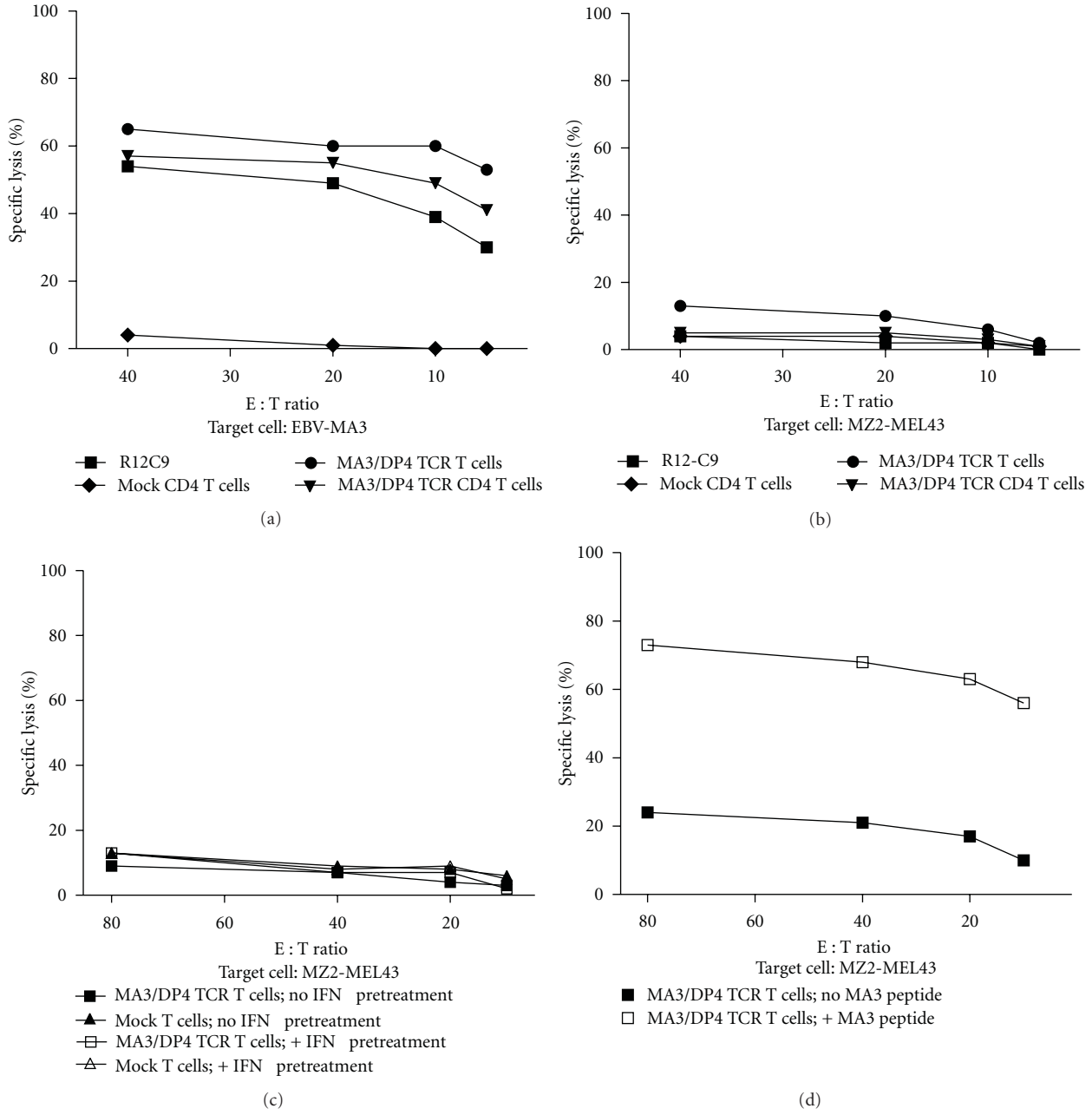

Figure 5: MA3/DP4 TCR T cells specifically lyse MA3-transduced or peptide-loaded B cells, but not MA3-positive melanoma cells. (a) MA3/DP4 TCR T cells specifically lyse DP4-positive B cells transduced with MA3-encoding cDNA. Human T cells were tested in a 6 h ^{51}Cr-release assay using EBV-MA3 target cells. The following effector T cells were used: CD4 T-cell clone R12-C9, MA3/DP4 TCR T cells, nondepleted T cells, MA3/DP4 TCR T cells depleted for CD8 T cells, or Mock T cells depleted for CD8 T cells. MA3-negative, DP4-positive B cells (BSM) were not recognized by MA3/DP4 TCR T cells (data not shown). (b) MA3/DP4 TCR T cells do not lyse MZ2-MEL43 melanoma cells, natively expressing MA3 and DP4. Effector T cells used were those described in legend to Figure (a). (c) MA3/DP4 TCR T cells do not lyse MZ2-MEL43 melanoma cells that are pretreated with IFNγ. Target cells were MZ2-MEL43 cells that were either pretreated with IFNγ or not, and effector T cells were MA3/DP4 TCR or Mock T cells. (d) MA3/DP4 TCR T cells lyse MZ2-MEL43 melanoma cells that are pulsed with MA3 peptide. Target cells were MZ2-MEL43 cells that were either pulsed with MA3 peptide or not, and effector T cells were MA3/DP4 TCR T cells. Measurements were performed in triplicate and expressed as mean values corrected for medium values. Data are from representative experiments out of 3 experiments with similar results.

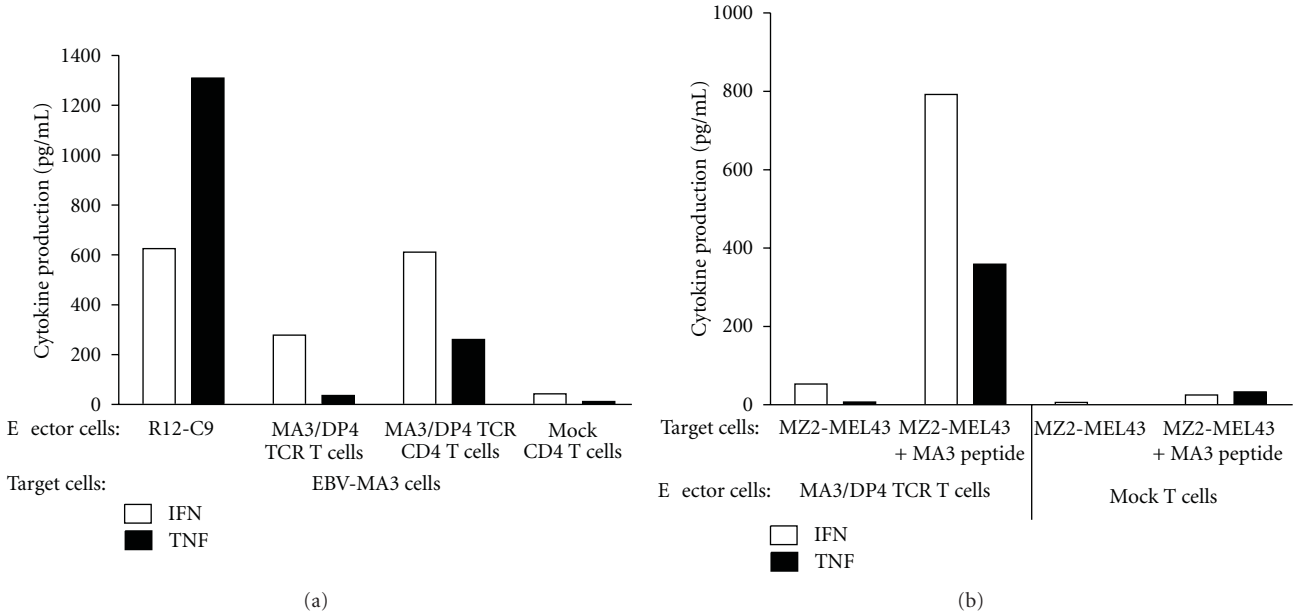

Figure 6: MA3/DP4 TCR T cells specifically produce IFNγ and TNFα upon coculture with MA3-transduced or peptide-loaded B cells, but not MA3-positive melanoma cells. Cytokine production is determined in supernatants of T cells after an 18 h co-culture with (a) DP4-positive B cells transduced with Ii-MA3 cDNA (EBV-MA3) or (b) MZ2-MEL43 cells loaded with MA3 peptide or not. In (a), effector T cells were: the CD4 T-cell clone R12-C9; MA3/DP4 TCR or Mock T cells, either nondepleted or depleted for CD8 T cells. MA3-negative, DP4-positive B cells (such BSM) were not recognized by MA3/DP4 TCR T cells (data not shown). In (b), MA3/DP4 TCR or Mock T cells, non-depleted, were used as effector T cells. Supernatants were harvested and analyzed for IFNγ and TNFα by ELISA. Measurements were performed in triplicate and expressed as mean values corrected for medium values. Data are from representative experiments out of 3 experiments with similar results.

4. Discussion

Redirection of T cells towards tumor-specific yet clinically safe antigens holds great promise for the treatment of melanoma and other tumor types. In the current paper, we have studied MAGE-C2/HLA-A2 (MC2/A2) and MAGE-A3/HLA-DP4 (MA3/DP4) as targets of TCR T cells; besides being prevalent in the patient population, these antigens are uniquely expressed by tumors and have proven value in initiating clinically effective CD8 and CD4 T-cell responses [23, 27, 28, 46].

MC2/A2 and MA3/DP4 TCR$\alpha\beta$ genes were derived from T-cell clones obtained from MAGE-vaccinated patients and were subsequently characterized following TCR gene transfer (see Figure 1). The CD8 T-cell clone EB81-CTL 16 expressed the MC2/A2-specific TCR-Vα3 and TCR-Vβ28 chains. Upon gene transfer, primary human T cells bound pMHC and demonstrated MC2-specific T-cell functions. TCR T cells killed and produced IFNγ and TNFα upon coculture not only with MC2 peptide-pulsed HLA-A2-positive target cells but also native MC2-positive, HLA-A2-positive melanoma cells (see Figures 2 and 3). T-cell responsiveness towards native MC2-positive, HLA-A2-positive melanoma cells (i.e., EB81-MEL-2 cells) was enhanced by IFNγ pretreatment, which promotes antigen processing and surface expression of MHC and adhesion molecules. In fact, unlike other antigenic peptides, the MC2 epitope ALKDVEERV (i.e., $MC2_{336–344}$) requires immune proteasomes for proper processing and presentation to T cells [52], supporting the value of MC2/A2 as a target for T-cell therapy. The effective dose of MC2/A2 peptide at which CD8 T cells demonstrate a half-maximal lytic response (i.e., ED50) is 0.75 nM [53]. This value represents a measure of T-cell avidity and lags somewhat behind in comparison to reported values for T cells expressing other MHC class I TCRs (range: 30–100 pM) [54, 55], suggesting a lower-to-intermediate ligand-binding affinity of this TCR. Experiments with mutated pMHC complexes that either prevent or enhance CD8α binding (according to [56, 57]; kindly provided by Professor Dr. Andrew Sewell, University of Cardiff, Wales) confirm the CD8-dependency of this MC2 TCR (data not shown).

The CD4 T-cell clone R12-C9 expressed the MA3/DP4 specific TCR-Vα38 and TCR-Vβ2 chains, which upon gene transfer in primary human T cells, resulted in pMHC binding (see Figure 4). In addition, MA3/DP4 TCR T cells, containing both CD8 and CD4 T cells (i.e., nondepleted), specifically lysed and produced IFNγ and TNFα upon coculture with B cells either transduced with MA3 antigen (Figures 5(a) and 6(b)) or loaded with MA3 peptide (data not shown). The extent of lysis, a typical measure for CD8 T cell function, was lowered when testing MA3/DP4 TCR CD4 T cells (i.e., depleted for CD8-positive T cells). The responsiveness of MA3/DP4 TCR T cells towards MZ2-MEL43 cells, generally weak and not reproducible, was not

enhanced by IFNγ pretreatment of target cells, whereas T cells were clearly able to recognize melanoma cells following loading with the MA3 16-mer peptide but not a core 12-mer peptide (TQGFVQENYLEY, i.e., $MA3_{247–258}$) (Figures 5(b), 5(c), 5(d), and 6(b)). Collectively, these data argue that MA3, like other nuclear proteins, may be inefficiently presented by tumor cells, and T-cell responses directed to tumor cells natively expressing MA3, such as those reported for T-cell clones 22 and R12–57 [58, 59] are rare and difficult to reproduce. In fact, when screening a panel of 23 T-cell clones including many patient R12-derived T-cell clones, we were unable to identify a single T-cell clone that responded towards MZ2-MEL43 (data not shown). In this respect, it is noteworthy that R12-derived CD4 T-cell clones show a polyclonal response towards MA3/DP4, with 50% of clonotypes sharing TCR-Vβ12 gene [46]. Thus, MA3/DP4 TCR T cells are able to lyse antigen-positive target cells, but lysis becomes suboptimal in case (low levels of) antigen is presented by tumor cells. The ED50 value of MA3/DP4 peptide in a CD4 T cell IFNγ assay is 30 nM [59]. This value is in accordance with reported values for T cells expressing other MHC class II TCRs (range: 40–200 nM) [20, 60]. Notably, functional expressions of MHC class II TCRs, such as reported for NY-ESO-1/DP4 TCR, may depend on the presence of the CD4 coreceptor [20], and are assessed by typical CD4 T cell assays, such as T cell proliferation and cytokine production. In case of MA3/DP4 TCR, we also observed that antigen-specific IFNγ production is higher in T cells depleted for CD8 T cells (i.e., CD4 T cells) when compared to nondepleted T cells (i.e., CD4+ CD8 T cells, Figure 6(a)).

Antitumor responses more typical for CD4 T cells are induced by professional antigen-presenting cells, such as DC (reviewed in [61]). DC capture and process tumor antigens and cross-present MHC class II-restricted antigens to CD4 T cells. Following activation, these CD4 T cells provide signals to DC that enhance antigen presentation and costimulation (via cross-linking of CD40) and lead to priming of antigen-specific CD8 CTL function [62]. Importantly, activated CD4 T cells are a major source of IFNγ, an effector cytokine with potent tumor regressing activity via inhibition of tumor-induced angiogenesis or activation of tumor-infiltrating macrophages [63–65]. When analyzing DC-induced T-cell responses, we observed significant production of cytokines when immature or mature DC were loaded with MA3 protein and used to stimulate TCR T cells (Figure 7). Decreasing the MA3 protein concentration during maturation of the DC from 25 to 5 μg/mL resulted in only slightly lower but almost comparable cytokine responses (data not shown). These findings extend the observations with two other MHC class II-restricted TCRs specific for human antigens, that is, NY-ESO1/DP4 and DBY/DQ5 [20, 66]. MA3/DP4 TCR-transduced T cells produced high amounts of IFNγ, whereas TNFα, IL-2, IL-4, and IL-5 were produced to a lesser extent. IL-10 represents the only cytokine with production levels being low (<20 pg/mL) and not different from TCR and Mock-transduced T cells (Figure 7). In addition, coculture with MA3-positive DC resulted in upregulated expression of T-cell activation markers such as CD25 (IL-2Rα chain) as well as enhanced T-cell proliferation (data not shown). Analysis of DC phenotype and function after coculture with MA3/DP4 TCR T cells was not possible, since DC died at days 1 and 2 after the start of coculture and were completely lost at day 4, which was evidenced by light microscopy and lack of IL-12 production and suggested direct killing of DC by TCR T cells. Our observation that MA3/DP4-specific CD4 T cells recognize MA3-protein-loaded DC rather than MZ2-MEL43 melanoma cells implies that these CD4 T cells yield antitumor activity *in vivo* following cross-presentation by professional antigen presenting cells. The therapeutic benefit of antigen-specific IFNγ production have initiated studies in which CD4 T cells were used as recipient T cells for MHC class I-restricted TCR. Not only can CD4 T cells be functionally endowed with MHC I-restricted TCR via gene transfer [19, 67, 68], but also can genetic cointroduction of CD8α skew TCR-engineered T cells towards an antigen-specific Th1 type T-cell response [69]. Vice versa, the introduction of a MHC class II TCR and CD4 coreceptor in CD8 T cells may lead to the generation of T cells with combined helper and effector T-cell functions [66].

In extension to our results with wild type MC2 and MA3 TCRs, we have tested gene optimization, a transgene cassette and another retroviral vector to enhance functional expression of TCR transgenes [70–72]. To this end, we have cloned codon-optimized MC2 and MA3 TCRs in TCRβ-2A-TCRα cassette-containing pMP71 vectors and demonstrated significant TCR surface expression and MAGE-specific IFNγ production by CD3 mAb-activated and transduced PBMC (note that results with pMP71: optTCRβ-2A-TCRα reflect bulk, nonsorted T cells, *Supplementary Figures 1 and 2*). In preparation of clinical studies, we propose the following additional strategies to enhance the therapeutic efficacy of T cells gene engineered with MC2 and MA3 TCRs. *First*, administration of common γ-cytokines, such as a combination of IL-15 and IL-21, to cultures of TCR T cells prior to patient infusion will yield T cells that show limited T-cell differentiation, and are better equipped to persist and function *in vivo* ([73]; Lamers, manuscript in preparation). *Second*, we propose preconditioning of patients that, apart from nonmyeloablative treatment with cyclophosphamide and fludarabine [1], includes treatment with the DNA hypomethylating agent 5-AZA-CdR. Such treatment, already used clinically, is reported to enhance expression of MAGE antigens and HLA in melanoma [26, 74]. And *third*, cotreatment with MC2 TCR-transduced CD8 and MA3 TCR CD4 T cells may be of particular interest to boost antitumor immunity and counteract selected growth of epitope-negative tumor variants. In fact, we have recently demonstrated that single-epitope targeting of melanoma by TCR-engineered T cells results in highly effective but transient regression in HLA-A2 transgenic mice and that more effective strategies likely require multi-epitope targeting (Straetemans, manuscript submitted). The proposed dual-epitope targeting approach may prove especially effective for CTA epitopes because of their coregulated expression pattern in tumor cells, with the vast majority of tumor cells expressing two or more CTAs [75]. Testing of cell lines derived from tumors other than melanoma has started in our

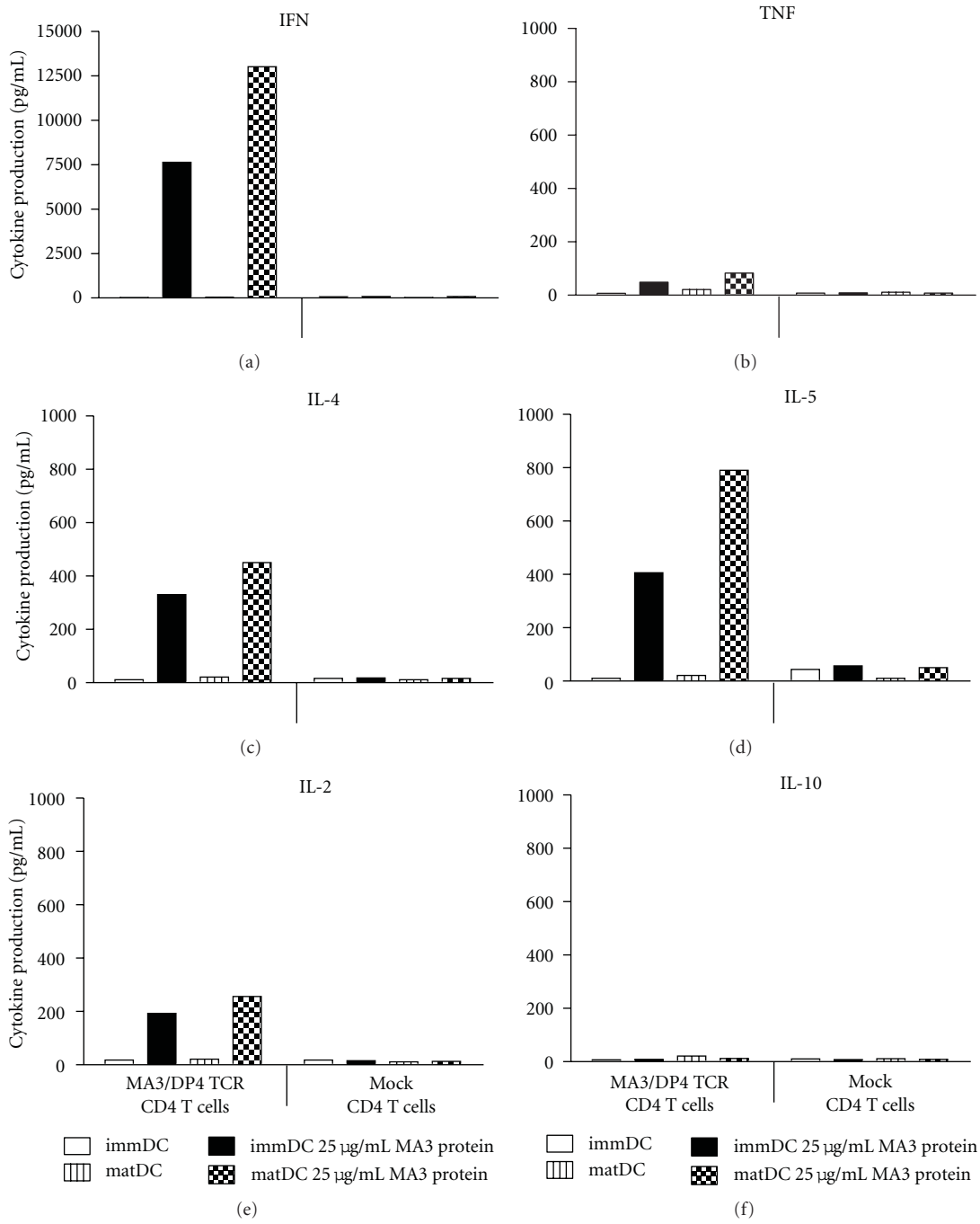

Figure 7: MA3/DP4 TCR CD4 T cells produce cytokines upon coculture with MA3 protein-loaded autologous dendritic cells. MA3/DP4 TCR, and Mock CD4 T cells were cultured with immature or mature autologous dendritic cells that were either loaded with 25 μg/mL MA3 protein or not. After 4 days, supernatants were harvested and analyzed for cytokine production by cytokine bead arrays. Cytokine production was not detected in case T cells were cultured without dendritic cells (data not shown). Measurements were performed in duplicate and expressed as mean values. Data are from a representative experiment out of 2 experiments with similar results.

laboratory, and may provide a preclinical rationale to extend the proposed treatment to nonmelanoma tumors.

In short, we have cloned and *in vitro* validated two MAGE-specific TCRs that warrant clinical testing in TCR gene therapy in melanoma patients and in other patients with cancers expressing the MC2 and MA3 antigens.

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Review Article

Cytotoxic Chemotherapy and CD4+ Effector T Cells: An Emerging Alliance for Durable Antitumor Effects

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Standard cytotoxic chemotherapy can initially achieve high response rates, but relapses often occur in patients and represent a severe clinical problem. As increasing numbers of chemotherapeutic agents are found to have immunostimulatory effects, there is a growing interest to combine chemotherapy and immunotherapy for synergistic antitumor effects and improved clinical benefits. Findings from recent studies suggest that highly activated, polyfunctional CD4+ effector T cells have tremendous potential in strengthening and sustaining the overall host antitumor immunity in the postchemotherapy window. This review focuses on the latest progresses regarding the impact of chemotherapy on CD4+ T-cell phenotype and function and discusses the prospect of exploiting CD4+ T cells to control tumor progression and prevent relapse after chemotherapy.

1. Introduction

As a major treatment modality for many advanced cancers, conventional chemotherapy can achieve high response rates but is rarely curative. The mounting evidence that many chemotherapeutic agents have immunostimulatory effects has provided a compelling rationale for developing combined chemoimmunotherapy strategy to achieve improved patient outcomes [1–3]. Current cancer immunotherapies predominantly rely on CD8+ T cells to fight against tumors. Although it is increasingly clear that proinflammatory CD4+ effector T cells are critical determinants of effective antitumor immune responses [4–9], the utilization of CD4+ T cell-based immunotherapy in combination with chemotherapy to control tumor progression and recurrence has not been fully explored. Nonetheless, a plethora of information accumulated from preclinical and clinical studies suggests that these two treatment modalities might be mutually reinforcing, and therefore their combination represents an effective chemoimmunotherapy strategy.

2. Anticancer Drugs and Immune Activation

Anticancer drugs are selected for their cytotoxicity toward cancerous cells. Although some anticancer drugs were known to have immune-potentiating effects long time ago [10, 11], the therapeutic potential of this property has been largely ignored. As increasing numbers of conventional chemotherapeutic agents are found to possess immunostimulatory properties, it has come to the realization in recent years that elicitation of the host antitumor immunity may constitute an integral component of the anticancer efficacy of some antineoplastic agents [12].

Multiple classes of anticancer chemotherapeutic drugs have been reported to exert immune enhancing effects, and a number of them have been extensively studied. Cyclophosphamide (CTX) is an alkylating agent chemically related to nitrogen mustard. As a prodrug, CTX is converted into its active metabolite derivative phosphoramide mustard in the liver. Phosphoramide mustard inhibits DNA replication by forming crosslinks between (interstrand) and

within (intrastrand) DNA strands. CTX is often used in combination with other anticancer drugs in the treatment of lymphomas and some solid tumors. Doxorubicin is a cytotoxic anthracycline antibiotic. It is known to bind to nucleic acids by intercalating the DNA strands and disrupting DNA replication. Doxorubicin is commonly used to treat hematological malignancies (leukemia, lymphoma, and multiple myeloma), and many types of solid tumors. Gemcitabine is a pyrimidine nucleoside analog that acts as an antimetabolite. Gemcitabine is used in a wide range of carcinomas, including lung, pancreatic, breast, and bladder cancer. Paclitaxel and docetaxel belong to the taxane class of drugs that act as mitotic inhibitors. These drugs cause cell-cycle arrest by stabilizing GDP-bound tubulin in microtubules, thereby disrupting the process of cell division. They are currently used to treat patients with lung, breast, prostate, and ovarian cancer. Cisplatin and oxaliplatin are platinum-based anticancer drugs. These platinum complexes induce apoptosis in malignant cells by causing crosslinking of DNA.

Although these anticancer drugs cause tumor destruction through different mechanisms, they share some common features in exerting immune-enhancing effects.

2.1. Inducing Immunogenic Tumor Cell Death. Tumor cells killed by anticancer drugs not only provide the source of tumor antigens but also release "danger signals" that awaken the innate immune cells, which in turn activate the adaptive immune system. Studies from Zitvogel's group have characterized several prominent features of immunogenic cell death after cytotoxic chemotherapy, including translocation of calreticulin (CRT), secretion of high-mobility-group box 1 (HMGB1), and release of adenosine triphosphate (ATP) by dying tumor cells. These studies reported that doxorubicin induces rapid translocation of the endoplasmic reticulum-resident protein calreticulin to tumor-cell surface, presenting a "eat-me" signal for phagocytosis by dendritic cells [13]. HMGB1 released by dying tumor cells after doxorubicin or oxaliplatin treatment acts upon toll-like receptor 4 (TLR4) on dendritic cells to initiate efficient antigen processing and presentation that involves the Myd88-signaling pathway [14]. Doxorubicin and oxaliplatin can also induce release of ATP by tumor cells, which triggers purinergic P2RX7 receptors on dendritic cells (DCs) to activate the NOD-like receptor family, pyrin-domain-containing protein 3-dependent caspase-1 activation complex, namely, the NLRP3 inflammasome, which ultimately leads to IL-1β-dependent adaptive immunity [15]. Along the same line, cyclophosphamide has been recently reported to cause CRT translocation and HMGB1 release in some types of tumor [16, 17]. Furthermore, it has been shown that tumor-cell apoptosis induced by gemcitabine can enhance DC cross-presentation of tumor antigen to CD8+ T cells [18], but it is not yet clear whether CRT translocation, mobilization of HMGB1, and ATP are involved in the process.

2.2. Mitigating Immunosuppressive Mechanisms. The ability of tumors to evade immune destruction is critical for tumor formation and progression and is now regarded as an emerging hallmark of cancer [19]. Under the selection pressure imposed by natural immune surveillance or therapeutic interventions, tumors may avoid immune attacks through passive mechanisms such as downmodulating the expressions of the relevant MHC-I molecules and antigens [20, 21]. In addition, tumor cells have evolved to employ multiple immune regulatory mechanisms to actively attenuate and subvert antitumor immune responses. Regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs) are frequently enriched in the tumor microenvironment and facilitate tumor immune evasion [22]. Some chemotherapeutic agents can potentiate antitumor immune responses by directly targeting these immunosuppressive cells. Low-dose cyclophosphamide (100 mg/kg) is capable of depleting cycling CD4+CD25+ Tregs and inhibiting their suppressive activity [23, 24]. As a result, the effector activities of cytotoxic CD8+ T cells and NK cells are unmasked to control tumor growth [25–27]. A recent study has suggested that CTX can preferentially deplete tolerogenic CD8+ lymphoid-resident DCs, leading to diminished Treg suppression and enhanced effector T-cell function as manifested by induction of concomitant immunity in a prophylactic setting [28]. It is currently unclear whether this mechanism of action is operative in a therapeutic setting. On a different note, gemcitabine does not deplete Tregs [24] but selectively reduces CD11b+Gr1+ MDSCs and enhances the antitumor activities of CD8+ T cells and NK cells [29].

2.3. Creating Lymphopenia and Immunogenic Milieu. Many anticancer drugs can cause varied degree of lymphodepletion [30]. It has been well established that lymphodepletion induced by chemotherapy or radiotherapy profoundly enhances the efficacy of adoptive cell therapy (ACT) and cancer vaccines [31]. This is likely due to the combined effects of creation of space and increased availability of stimulatory growth factors that lead to enhanced proliferation and survival of activated T cells [32]. In this regard, cyclophosphamide is a representative anticancer drug that causes profound lymphodepletion while creating an immune milieu rich of type I interferons (IFNs) and common gamma-chain cytokines (IL2, IL7, and IL15) [33, 34]. Of notice, type I interferons are known to promote DC maturation and T-cell differentiation [35–38]. IL7 is essential for survival and memory formation of tumor-reactive T cells, and neutralization of IL7 after CTX administration diminishes the number of tumor-reactive T cells in an adoptive transfer model [33]. Besides strengthening the activities of immune cells, chemotherapy also promotes the trafficking of activated immune cells to the sites of tumor [33, 39, 40]. Accumulating evidence demonstrates that there is a surge of proinflammatory cytokines/chemokines, such as GMCSF, IL1β, IL6, and CXCL10, in the postchemotherapy immune milieu, which may contribute to the recruitment and retention of tumor-reactive immune cells, including activated CD8+ and CD4+ T cells, DCs, macrophages, and neutrophiles, in the tumor microenvironment [15, 17, 34].

2.4. Sensitizing Tumor Cells to Immune Destruction. In addition to attracting activated immune cells to the tumor loci, chemotherapy may render tumor cells more susceptible to immune attack. It has been shown that doxorubicin, cisplatin, and paclitaxel can sensitize tumor cells to the cytolytic effect of CD8+ T cells by making them permeable to granzyme B via mannose-6-phosphate receptors on the surface of tumor cells [40]. Moreover, chemotherapy with cyclophosphamide can sensitize tumor cells to TRAIL-dependent CD8+ T cell-mediated immune destruction [41].

3. Chemotherapy and Antitumor CD4 Responses

A great deal of effort has been focused on understanding how chemotherapy potentiates CD8+ T-cell responses [27, 38, 41, 42], mitigates Treg-mediated immune suppression [23, 26, 43], and enhances antigen presentation [13, 14, 28, 44, 45]. Although tumor-reactive CD4+ effector/helper T cells are increasingly recognized as critical determinants of effective antitumor immune responses, the effect of chemotherapy on these cells is largely neglected, and the role of CD4+ T cells in modulating postchemotherapy host immunity is almost entirely unknown. In the following we mainly focus on findings that concern the impact of chemotherapy on the interactions between tumors and CD4+ T cells.

3.1. CD4+ T Cells Subsets and Their Diverse Roles in Tumor Immunity. Upon stimulation naïve CD4+ T cells differentiate into effector cells known as T helper (Th) cells. Originally Th cells were classified into Th1 and Th2 lineages, depending on the cytokine profiles of the effector cells [46]. With the discovery of new T-cell lineages in recent years, the Th1/Th2 paradigm has been revised to reflect a much broadened spectrum of CD4+ T-cell subsets. It has now been established that naïve CD4+ T cells can differentiate into four major lineages, including Th1, Th2, Th17, and Treg cells [47], and that Th cells are plastic—cells of one lineage can be converted to another lineage under certain circumstances [48].

The distinct CD4+ T-cell subsets have varied impact on tumor growth. Th1 cells, characterized by production of IFNγ and TNFα, often lead to enhanced activation of cytotoxic CD8+ T cells, DCs and macrophages, exhibiting beneficial antitumor effects. In contrast, IL4-producing Th2 cells may promote tumor progression by enhancing the activity of protumor macrophages [49] although Th2 cells can also mediate tumor rejection under certain condition [50]. Currently there is much debate about the role of Th17 cells in antitumor immunity [51], because both tumor rejection and tumor promotion involving Th17 cells and their major product proinflammatory cytokine IL17 have been reported [52–55]. Treg cells act to dampen antitumor immunity by suppressing the effector functions of a variety of immune cells, including Th1 cells [56–58], CD8+ T cells [5], NK cells [59], and tumor-infiltrating DCs [60].

3.2. Effect of Chemotherapy on CD4+ T-Cell Effector Development. So far, among the aforementioned anticancer drugs, cyclophosphamide (CTX) appears to be the most effective one in enhancing antitumor CD4 responses, particularly when used in combination with adoptive cell therapy (ACT). It has been demonstrated in various preclinical models that CTX treatment followed by adoptive transfer of tumor-reactive CD4+ T cells, either monoclonal T-cell clones derived from TCR-transgenic mice, or activated polyclonal CD4+ T cells derived from preimmunized mice, leads to eradication of established tumors [61–64]. One salient observation from these studies is that the robust antitumor effects are associated with the development of Th1 antitumor immunity. In line with an early study showing that CTX induced a Th2 to Th1 shift in the cytokine profile of lymphoma-bearing rats [65], we have recently reported in a mouse lymphoma model that CTX overcomes tumor-driven aberrant CD4+ T-cell differentiation and directs CD4+ T cells to become highly activated polyfunctional effector cells, marked by their ability to concomitantly produce multiple Th1-type cytokines including IL2, IFNγ, and TNFα [64]. In a mouse melanoma model, Quezada et al. reported that tumor-specific CD4+ T cells acquired a similar polyfunctional phenotype in postradiotherapy hosts [66], suggesting that the immunogenic milieu created by chemotherapy or radiotherapy may share some common features in terms of driving CD4+ T-cell effector differentiation. In addition to promoting Th1 differentiation, there is emerging evidence that CTX also induces Th17 cells [34, 67]. These Th17 cells are likely de novo induced in the postchemotherapy milieu, because they are not converted from Treg cells [67], and do not coexpress IFNγ [34]. In contrast, doxorubicin and oxaliplatin each induces IL17-producing $\gamma\delta$T cells but not Th17 cells [68]. It will be of interest to test additional anticancer drugs to define the common features of the drugs that are capable of driving effector CD4 responses like CTX.

3.3. Mechanisms by Which Anticancer Drugs Modulate CD4 Responses. Even though CTX is by far the most potent CD4-potentiating anticancer drug demonstrated experimentally, the cellular and molecular mechanisms underlying its effect are not well understood. In addition to its well-known effect of depleting suppressor T cells, accumulating evidence has established a link between productive CD4+ T-cell responses and an immunogenic milieu induced by CTX [17, 33, 36, 64]. The immunogenic milieu is rich of various growth factors and proinflammatory cytokines and chemokines, among which type I IFNs and IL7 have been shown to exert particularly important immunostimulatory effects. Type I IFNs can augment immune responses through enhanced stimulation of dendritic cells [69]. It has been shown that DCs require type I IFNs to mature and induce CD4+ Th1 immunity [70]. In the same vein, a recent study has reported that IFNα enhances T helper cell functions while reducing Treg activity through modulating APC activation [71]. In addition to supporting T-cell survival and homeostasis, IL7 has recently been shown to antagonize cbl-b and TGFβ signaling, two pathways involved in inhibiting T-cell activation, leading to

augmented Th17 differentiation [72]. Moreover, it has been reported that IL7 promotes Th1-like immunity and inhibits Treg activity [73, 74]. Altogether it is conceivable that CTX's multifaceted and dynamic immunomodulatory effects, for example, depletion of Treg, creation of lymphopenia, and induction of stimulatory cytokines superimpose to foster a profoundly immunogenic milieu that drives the development of fully differentiated Th1 or Th17 effector T cells. To better understand the mechanisms underlying the diverse CD4+ T-cell differentiation in postchemotherapy setting, future studies should dissect the interrelation of the abovementioned contributing factors, and their relative contribution to the functional development of tumor-specific CD4+ T cells.

3.4. Antitumor Effects of CD4+ Effector T Cells

3.4.1. Activating Tumoricidal CD8 and Macrophages and Sensitizing Tumor Stroma. CD4+ T cells have been regarded as specialized helper cells that assist in the activation of other innate and adaptive immune cells. Once properly activated, CD4+ T cells express an array of effector molecules, including CD40L, IL2, IFNγ, and TNFα, which play critical roles in orchestrating effective antitumor immune responses. Consistent with the well-defined role of CD40L in transmitting CD4 help for CD8+ T cells [75–77], it has been shown in different animal models that activated CD4+ T cells can license DCs in the tumor microenvironment via CD40L-CD40 interaction, leading to priming of tumor-reactive CD8+ T cells which in turn mediate long-term protection [78, 79]. In addition to licensing of DC, some previously unappreciated help activities of CD4+ T cells have recently been uncovered, revealing the molecular basis of the once vaguely-defined "post-licensing" role of CD4+ T cells [80]. For example, it has been shown that CD4+ effector T cells recruit activated CD8+ T cells via the action of IFNγ [81, 82] and promote CD8+ T-cell cytolytic function and proliferation through IL2 [82]. Besides targeting tumor cells, CD4+ effector T cells have been implicated in inhibiting tumor angiogenesis by acting on tumor stroma via IFNγ [83]. Given that CD8+ T cell-derived TNFα and IFNγ can sensitize tumor stroma and mediate bystander tumor eradication [84], we speculate that polyfunctional CD4+ effector T cells have the same effect because these cells can produce these two cytokines simultaneously [34]. Notably, it has been reported that Th1-derived IFNγ also renders macrophages cytotoxic to cancer cells [6, 85]. Interestingly, Beatty et al. reported that CD40-activated macrophages become tumoricidal and facilitate the destruction of tumor stroma in mice and humans with pancreatic carcinoma [86]. Although this study used an agonist CD40 antibody to activate macrophages, it is tempting to speculate that CD40L-expressing CD4+ effector T cells would achieve similar effects.

3.4.2. Conditioning a Protective Inflammatory Milieu. Chemotherapy often induces inflammation in the tumor microenvironment by causing tumor cell death and tissue damage. Paradoxically, many of the proinflammatory cytokines induced after chemotherapy, particularly IL1β, IL6, and IFNα/β, can exert both tumor-inhibiting and tumor-promoting effects (double-edged sword) [87, 88]. On one hand, IFNα/β and IL1β both can directly act on CD4+ T cells to enhance their activation and differentiation [89–92]. In addition, IFNα/β and IL1β can augment antigen presentation and facilitate priming of T cells [15, 37, 70, 93]. Moreover, IL6 and IFNα/β can potentiate effector cells to resist Treg-mediated suppression [71, 94], and IL6 and IL1β can mediate Treg $\rightarrow$ Th17 conversion [95–97]. On the other hand, IL1β and IL6 have been shown to drive tumorigenesis [98–103] and dampen host immunity by expanding myeloid-derived suppressor cells (MDSCs) [104–106]. IFNα/β are potent inducers of coinhibitory molecules PDL1 [107] and PD1 [108], and immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) [109, 110]. Furthermore, it has been shown that IL6 contributes to chemoresistance [111]. Intriguingly, the efficacy of many cancer therapies is often associated with certain degree of inflammatory responses [34, 112, 113]. A recent study by Haabeth et al. has suggested that unopposed inflammation may promote tumor progression while the presence of Th1 cells can tilt inflammation toward effective antitumor immunity [85]. This hypothesis is supported by the observation that chronic inflammation associated with psoriasis, a Th1-mediated autoimmune disease affecting the skin, does not promote the development of skin cancers [114]. Therefore, it is likely that Th1 CD4+ T cells play a critical role in conditioning a tumor-inhibiting inflammatory milieu that facilitates immune activation and tumor destruction.

3.4.3. Mediating Direct Tumor Destruction. Besides rendering other immune cells tumoricidal, CD4+ T cells have the capability to mediate direct tumor destruction. It has been shown that CD4+ T cells can induce apoptosis in tumor cells through FAS- or TRAIL-dependent pathway [115, 116]. Moreover, there is accumulating evidence that CD4+ T cells can acquire cytolytic activities like cytotoxic CD8+ T cells [117–121]. However, the significance of this property has been largely ignored, until recently two studies have provided compelling evidence that cytotoxic CD4+ T cells developed in a lymphopenic environment can eradicate established melanoma as a result of direct killing of the tumor cells through granzyme B [66, 122]. Currently it is unknown whether cytotoxic CD4+ T cells and helper CD4+ T cells develop in parallel, or they are the same cells at different stages of differentiation. Nevertheless, Qui et al. provided evidence that costimulation through CD134 (OX40) and CD137 (4-1BB) is required to drive the differentiation of cytotoxic CD4+ effector cells in an eomesodermin-dependent manner [123]. Although cytotoxic CD4+ and CD8+ T cells appear to mediate tumor killing using the same effector molecules, such as granzyme B and perforin, they target MHC-II and MHC-I-restricted antigens, respectively. One important implication of CD4+ T-cell cytotoxicity is that CD4+ T-cell-mediated tumor destruction may result in antigen spreading, which is associated with broadened antitumor CD8 responses and improved clinical responses [8, 124–126].

In summary, with an arsenal of diverse cancer-fighting weapons, CD4+ T cells can mediate tumor destruction either on their own or by cooperating with other immune cells. Whereas CD4+ T cells alone clearly have the potential to effectively eradicate tumors [66, 122, 127], the majority of published studies indicate that the optimal antitumor effects are achieved when CD4+ T cells act in concert with tumor-reactive CD8+ T cells [8, 78–80, 128–133], macrophages [6], or NK cells [7]. A long-held perception is that CD4 antitumor immunity is only relevant to the treatment of MHC-II$^+$ tumors. Nevertheless, due to the wide-range mode of actions, CD4+ T cells have been shown to play active and indispensable roles in controlling both MHC-II$^+$ [63, 64] and MHC-II$^-$ tumors [6, 7, 78, 79, 127, 134, 135]. It is worth noting that some solid tumors, melanoma, for instance, can be induced to express MHC-II upon encountering IFNγ and thus become direct targets of CD4+ effector T cells [66, 122]. Therefore, the generation of effective CD4+ T-cell responses has great therapeutic potential and broad clinical relevance.

4. Inhibitory Mechanisms That Attenuate Antitumor CD4+ T-Cell Responses

Tumor-specific CD4+ T cells are subject to a variety of tolerizing mechanisms operative in the tumor microenvironment. Induction of anergy in antigen-specific CD4+ T cells is an early event in the course of tumor progression [136]. We and others show that tumor-antigen recognition is accompanied with induction of both CD4+ effector cells and Tregs [56, 57, 137]. However, the tolerogenic nature of the tumor milieu progressively renders CD4+ effector T cells dysfunctional, characterized by sustained expression of PD1 and heightened apoptosis [64]. Thus, the anergic phenotype of the overall CD4 population represents the net result of Treg induction, effector dysfunction, and active immune suppression. Treg cells enriched in tumor may come from expansion of preexisting Tregs, and de novo induction of Treg cells [137, 138], which may occur in both antigen-dependent [138] and -independent [139] manner in tumor-bearing hosts. Pertaining to combinatory chemoimmunotherapy, it will be important to determine if highly activated CD4+ effector T cells are susceptible to Treg conversion in the face of minimal residual disease after chemotherapy. Although it has been shown that polarized Th1 effector cells and memory CD4+ T cells are refractory to conversion to Tregs [140, 141], whether this is the case in the postchemotherapy setting is yet to be addressed.

Tregs have been shown to attenuate antitumor responses through a variety of mechanisms, including deactivating DCs [142, 143], preventing CD8+ T-cell-mediated cytolysis [144], and direct killing of DC, NK, and CD8+ T cells [145, 146]. Importantly, Tregs may operate in concert with other regulatory mechanisms, including MDSC, coinhibitory molecule PD1, and immunosuppressive enzyme IDO, to form a self-reinforcing immunosuppressive network, posing a severe threat to the magnitude and durability of an effective antitumor immune response. MDSCs can act as tolerogenic APCs to expand Tregs [147, 148]. IDO+ DCs can directly activate Tregs which subsequently mediate suppression in a PD1/PDL1-dependent fashion [149]. Programmed death 1 (PD1) was initially found to mediate CD8+ T-cell functional exhaustion during chronic viral infections [150]. Subsequent studies confirmed the existence of exhausted PD1^{high} CD8+ T cells during tumor progression [151, 152]. However, the role of PD1 in regulating CD4+ T-cell response in the tumor context is less clear. Using a mouse B-cell lymphoma model, we provided clear evidence that PD1^{high} CD4+ T cells constituted a fraction of tumor antigen-experienced cells and were associated with downregulation of IL7 receptor and elevated level of apoptosis [64]. Interestingly, we showed in this model that PD1 was not required for tumor-driven Treg induction, while two other studies reported that PDL1 was involved in peripheral Treg induction and maintenance [153, 154]. Given that PD1 is not the only receptor for PDL1 [155–157], the seemingly discrepant results suggest that PDL1 on DCs may differentially regulate Treg induction and effector T-cell dysfunction through engaging different receptors on CD4+ T cells. This is supported by the observation that PD1 and Foxp3 have a nonoverlapping expression pattern in CD4+ T cells infiltrating B-cell lymphoma [158, 159]. Collectively, these findings and the results from other studies [160–163] support a scenario in which Treg-mediated suppression and PD1-dependent T-cell dysfunction contribute independently but synergistically to the failed immunological control of tumor growth.

5. Implications for Combined Chemoimmunotherapy

Standard chemotherapy is a major treatment option for many types of cancer. It can effectively treat the symptom of cancer initially, but frequently its efficacy is compromised by late tumor recurrence. The ability of some anticancer drugs to drive productive CD4+ T-cell responses, and the versatile and pivotal roles of CD4+ effector T cells in mediating antitumor effects, provide strong rationales for developing a strategy that utilizes CD4+ effector T cells to strengthen and sustain the postchemotherapy antitumor immunity. This can be achieved clinically through the combination of chemotherapy and adoptive immunotherapy or therapeutic vaccination. Indeed, the efficacy of this strategy has been hinted by some elegant clinical studies, which showed that better immunological and clinical responses were obtained in melanoma or myeloma patients that had received CD4+ T cell-containing donor cells following preconditioning chemotherapy [129, 131].

To overcome tumor-induced immune tolerance, additional maintenance regimens are needed to keep CD4+ T cells in the polyfunctional effector state. Many of the currently available immune modulators [164], such as recombinant IL7, CD40 agonist, PD1 blockade, and CTLA4 blockade, can be applied to potentiate and sustain CD4 effectors in addition to enhancing antitumor CD8 responses. We showed that polyfunctional CD4+ T cells have the unusual distinguishing attribute of high levels of IL7 receptor expression [64], suggesting that these cells can be preferentially

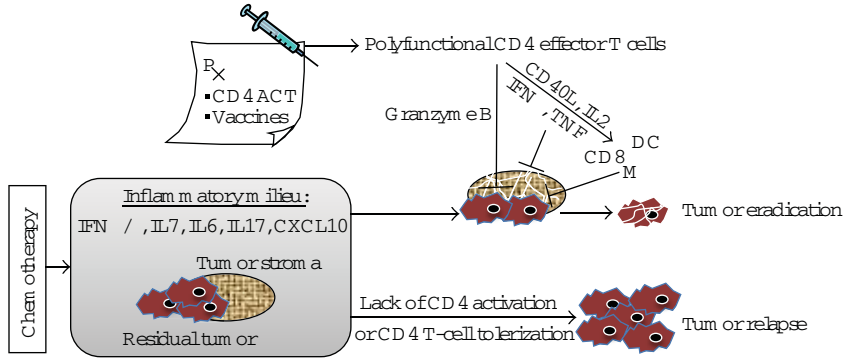

Figure 1: Hypothetical model of the mutually reinforcing effect of chemotherapy and antitumor CD4+ effector T cells. Chemotherapy reduces tumor burden, releases tumor antigens, and induces inflammation. In this highly immunogenic milieu created after chemotherapy, therapeutic immunological maneuvers such as adoptive cell therapy (ACT) using tumor-reactive CD4+ T cells or cancer vaccines can lead to the generation of highly activated CD4+ effector T cells with polyfunctional activities. These CD4+ effector T cells act as the "gatekeepers" of the overall antitumor immunity in postchemotherapy hosts, by helping the activation and function of other immune cells (CD8, DC, and macrophage) and directly attacking the tumor cells. In addition, cytokines produced by CD4+ effector T cells (IFNγ and TNFα) may also target and destroy tumor stroma and thus inhibit tumor angiogenesis. These diverse immune responses superimpose to effectively eradicate residual tumors. In contrast, without properly activated CD4+ effector T cells, an effective host antitumor immunity may not be elicited or is not sustainable, leading to tumor persistence and eventual relapse.

expanded by supplying exogenous IL7. Moreover, activating DCs with an anti-CD40 agonist antibody can prevent CD4+ T-cell tolerance [165]. PD1 blockade, currently undergoing extensive clinical trials for a variety of cancers [166, 167], is largely expected to restore CD8+ T-cell antitumor function but may as well benefit CD4+ effector T cells. Notably, CTLA4 blockade with ipilimumab, recently approved by FDA for the treatment of late-stage melanoma, has been shown to promote the generation of polyfunctional CD4+ T cells in response to vaccination [168].

With regard to alleviating Treg-mediated immunosuppression, current approaches only have limited success in therapeutic settings. Low-dose CTX reduces and inactivates Tregs, but doing so only transiently. Application of denileukin diftitox (Ontak) did not result in consistent clinical outcomes [169, 170], likely due to its effect on both effector T cells and Tregs. Findings from some recent studies suggest new strategies for disarming Tregs. It has been shown that combined use of CTX and an agonist antibody targeting the costimulatory receptor OX40 can result in intratumoral apoptosis of Tregs [42]. Moreover, Sharma et al. reported that disrupting the IDO pathway with clinically applicable pharmacological inhibitors can reprogram Tregs to Th17 cells [171].

Altogether, a successful combined chemoimmunotherapy should integrate strategies that target multiple mutually reinforcing immune pathways that converge to attain productive CD4 effector responses, thereby maintaining a durable and effective antitumor immunity after chemotherapy.

6. Conclusions

Although the concept of combined chemoimmunotherapy for cancer can be dated back to at least three decades ago [10, 11], its clinical application started to gain momentum only in recent years when the mechanistic basis for the synergy between chemotherapy and immunotherapy began to be unveiled at the cellular and molecular level. The emerging evidence that chemotherapy can profoundly drive the effector development of tumor-specific CD4+ T cells implicates a new direction for chemoimmunotherapy, which aims to capitalize on the antitumor potential of CD4+ effector T cells. In light of the unique and pivotal roles of tumor-reactive CD4+ effector T cells, we propose a scenario in which CD4+ effector T cells act as the "gatekeepers" of the overall host antitumor immunity after chemotherapy, whose functional status (polyfunctional versus tolerized) critically determines the outcome between eradication versus recurrence of residual tumors (Figure 1). Further studies are needed to explore additional CD4+ T cell-potentiating anticancer drugs and establish clinically applicable strategies for maximum utilization of the synergy between chemotherapy and antitumor CD4 effector responses in order to achieve durable therapeutic efficacy.

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Review Article

Modulation of Tumor Tolerance in Primary Central Nervous System Malignancies

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Central nervous system tumors take advantage of the unique immunology of the CNS and develop exquisitely complex stromal networks that promote growth despite the presence of antigen-presenting cells and tumor-infiltrating lymphocytes. It is precisely this immunological paradox that is essential to the survival of the tumor. We review the evidence for functional CNS immune privilege and the impact it has on tumor tolerance. In this paper, we place an emphasis on the role of tumor-infiltrating myeloid cells in maintaining stromal and vascular quiescence, and we underscore the importance of indoleamine 2,3-dioxygenase activity as a myeloid-driven tumor tolerance mechanism. Much remains to be discovered regarding the tolerogenic mechanisms by which CNS tumors avoid immune clearance. Thus, it is an open question whether tumor tolerance in the brain is fundamentally different from that of peripheral sites of tumorigenesis or whether it simply stands as a particularly strong example of such tolerance.

1. Introduction

Central nervous system (CNS) tumors account for high rates of morbidity and mortality [1]. In children, CNS tumors represent the most common solid tumors with more than 3100 newly diagnosed patients in the United States annually [2]. Overall 5-year survival statistics are a dismal 35% in adult patients while they approach 75% in the pediatric population [1, 2], likely owing to fundamental differences in tumor biology. Even so, more children die each year from brain tumors—more than 2700 per year [2]—than from any other cancer. Patients with aggressive CNS tumors (glioblastoma multiforme, diffuse intrinsic pontine glioma, atypical teratoid/rhabdoid tumor, etc.) fare particularly poorly due to the high grade infiltrative nature of their disease and fundamental resistance to radiotherapy and current chemotherapy regimens. In fact, patients with high grade gliomas generally succumb to progression of persistent or recurrent disease [3].

CNS tumors may have a specialized immune biology that allows evasion of immune clearance and promotion of tumor-growth, and the tissue milieu within which a CNS tumor naturally grows may be especially important to supporting this immunobiology. The term "immune privilege" has been used to describe deficient or defective adaptive immune responses that translate to an absence of tumor-specific immune responses (Table 1). Treatment of brain cancers is further complicated by the presence of a small molecule exclusion system, the blood-brain barrier, which limits the CNS penetration of many chemotherapeutics. Despite the complexity of this blood-brain barrier, however, it does not block lymphocytes or myeloid cells from migrating to sites of inflammation or tumor growth [4, 5].

In fact, brain tumors contain large numbers of tumor associated macrophages (TAMs) and microglia as well as tumor infiltrating lymphocytes. These cellular components of the immune system apparently coexist with the developing tumor, and while antitumor responses are possible within the CNS [80], they are typically ineffective [38, 81–83]. In fact, the privileged status that brain tumors enjoy with respect to immune responses appears to be driven by highly active and dominant local immune suppression [38, 81, 83], as is

Table 1: Mechanisms of immune privilege.

General peripheral tolerance	Ref
T cell negative selection in thymus	[6]
Natural (thymic) Tregs	[7, 8]
Acquired (adaptive) Tregs	[9, 10]
Local immunosuppression (IDO, TGF-β, IL10, CTLA-4)	[11–27]
CNS-specific privilege	**Ref**
Reduced lymphatic transport to draining lymph nodes	[28–33]
Lack of resident immunogenic APCs (dendritic cells)	[28, 29, 34–37]
Specialized endothelium excludes naïve T cells	[28, 29, 34, 35, 38]
Local immunosuppression by astrocytes and microglia	[28, 35, 38–41]
Tumor-induced immunosuppression (CNS and non-CNS)	**Ref**
Local activation of natural Tregs	[7, 42–44]
Tumor-specific (adaptive) Tregs	[42, 44–48]
Local intratumoral immunosuppression	
IDO	[16, 45, 47–53]
Arginase	[42, 54, 55]
TGF-β	[56–59]
IL10	[60, 61]
CTLA-4	[11, 62–65]
PD-L1	[11, 46, 62, 66]
Myeloid-derived suppressor cells	[67–70]
Tolerogenic APCs	[42, 44, 45, 47, 48, 61, 71–73]
Tolerogenic draining lymph nodes	[45, 47, 48, 73]
Quiescent vascular endothelium	[74–79]

Tregs: regulatory T cells; IDO: indoleamine 2,3-dioxygenase; TGF-β: transforming growth factor-beta; IL10: interleukin-10; CTLA-4: cytotoxic T lymphocyte antigen-4; PD-L1: programmed death ligand-1; APCs: antigen-presenting cells.

the case with peripheral solid tumors [45]. However, in CNS tumors, we speculate that this local tumor-specific tolerance may be augmented by the specialized mechanisms of CNS privilege [28, 39, 84, 85]. Gaining a better understanding of these tolerogenic mechanisms is critically important to improving the survivability of malignant CNS tumors, which currently resist our most aggressive and multimodal therapeutic strategies.

2. Immune Privilege in the Central Nervous System

2.1. The Immune Privilege Paradigm. Uncontrolled immune responses in the brain are more dangerous than in any other location, and the central nervous system enjoys a distinctly different immunology than peripheral tissues [29, 34, 35]. Classical CNS "privilege" was described phenomenologically in terms of diminished or absent immune responses [29, 35]; particularly compelling was Medawar's observation that tissue graft rejection was impaired in the brain [86]. Additional findings suggesting a unique immunology existed in the CNS included lack of lymphatic vessels and lymph nodes within the CNS [29], lack of dendritic cells resident within the brain parenchyma [29], low major-histocompatibility (MHC) expression levels on all cells within the CNS—including low MHC-II on resident microglia [29, 35], and widespread presence of soluble anti-inflammatory mediators, such as vasoactive intestinal peptide [35], alpha melanocyte stimulating hormone [35], and transforming growth factor-beta (TGF-β) [35, 38]. Furthermore, production of inflammatory cytokines and nitric oxide by CNS resident myeloid cells, including macrophages and microglia, is suppressed by a cell-contact mediated receptor ligation to CD200, a ligand expressed by brain parenchymal cells [35]. Thus, the character and strength of immune responses in the CNS are fundamentally different than in the periphery. Presumably, these strict regulatory mechanisms [42] have evolved to preserve the nonrepairable brain tissue and avoid unchecked inflammation in a closed space that could otherwise lead to increased intracranial pressure, herniation, and death [29].

2.2. Leukocyte Entry into the Central Nervous System. Naïve T cells are effectively excluded from brain parenchyma by the tight junctions of the blood-brain barrier [28]. Thus, leukocyte trafficking generally occurs at very low frequency in quiescent brain [35]. Nonetheless, all the elements of an effective immune response—including dendritic cells, macrophages, and T cell lineages—can and do traverse the blood-brain barrier in inflammatory states [4, 29, 34, 35]. T cells usually become activated in extra-CNS sites, where they encounter an appropriate antigen before migrating into the CNS itself [29, 35]. T cells expressing the chemokine receptor CCR7 home effectively to the CNS via chemokine-mediated (CCL19 and/or CCL21) homing [29]. Leukocytes thus recruited enter the CNS at postcapillary venules by the standard process of tethering, leukocyte rolling, chemokine activation, adhesion, and diapedesis [29, 34, 38]. However, in the CNS, diapedesis appears to occur via transendothelial extravasation, rather than a paracellular route, which leaves the blood-brain barrier endothelial tight junctions intact [29, 85]. Once they have transmigrated through the vascular endothelium, these leukocytes find themselves in an enlarged perivascular space, the Virchow-Robin space [29, 34]. It is within this space that they will either encounter antigen to maintain their activated state, or fail to do so and die. To reach the CNS parenchyma, leukocytes still need to cross the *glia limitans* which is defined by the interlocking perivascular astrocyte foot processes [34]. Once in the CNS, however, activated T cells are free to carry out their effector functions [35].

2.3. Antigen Presentation in the Central Nervous System. A unique anatomical facet of CNS immunology is the lack of local draining lymph nodes. In fact, animal experiments have shown that labeled dendritic cells injected directly into CNS parenchyma do not appear to migrate from the

site of inoculation [30], whereas dendritic cells (DCs) in the interstitial fluid of the CNS behave more like DCs in peripheral sites and are able to migrate to the cervical lymph nodes via perivascular channels [31]. Other studies have shown that rat dendritic cells and microglia injected into the striatum migrate to the perivascular space and exit through the vasculature to reach distant sites, such as spleen and mesenteric lymph nodes [32]. In contrast, dendritic cells injected into the cerebral spinal fluid (CSF) migrate to the B cell follicles of cervical lymph nodes [30], and they do so by traversing the cribriform plate to reach the nasal lymphatics [31]. This is similar to experiments in which radio-labeled protein infused into the CSF preferentially drains to deep cervical lymph nodes via the cribriform plate [28, 33]. Thus, the afferent arm of local CNS immune surveillance is quite complex and, in some contexts, may bypass traditional lymphatic routes of antigenic sampling.

Not only do antigen-presenting cells (APCs) in the brain often fail to migrate into lymph nodes, but the CNS is also the only tissue with microglia as antigen-presenting cells, which imparts a unique immune biology to CNS-directed responses [36]. Microglia are derived from early monocytic cells during embryonic development [34, 37]. In adults and children, they can be replenished from progenitor cells in the CNS that have proliferative capacity for microglial renewal [34]. Microglia resemble resident perivascular macrophages with similar phenotypic markers and functional profiles [37]. Although resting microglia have a quiescent phenotype with low expression of MHC and costimulatory molecules, they have very dynamic motility, presumably consistent with their antigen-surveillance function [36]. In fact, effective responses to viral encephalitis depend upon microglial cytokine-mediated macrophage recruitment [34]. This cytokine production can lead to capillary leak and compromise the integrity of the blood-brain barrier [34], but such a breach will also cause local microglial activation and recruitment of circulating immune cells [36]. Thus, microglia play an important regulatory role in initiating responses to CNS infection and in modulating and directing intracranial immune responses.

3. Immune Privilege in the Setting of Central Nervous System Malignancy

3.1. General Events in Tumor Formation, Growth, and Survival. Tumors must develop complex stromal networks that promote vigorous growth but suppress adaptive immune responses—and tumors must accomplish this despite the presence of many intratumoral innate immune cells and tumor infiltrating lymphocytes [87, 88]. The stromal content of solid tumors is very large [89] (sometimes more stromal cells than tumor cells) and the paradoxical ability of this stroma to support growth yet suppress immune rejection is essential to the survival of the tumor.

3.1.1. Important Factors in Oncogenesis. Malignant transformation occurs when a critical mass of genomic and epigenetic mutations leads to uncontrolled cell division, either dominated by a loss of cell cycle control [90–93] or by a defect in apoptotic pathways [92, 94–96]. This results in a cluster of neoplastic cells, derived from a single progenitor, which grow without the constraint of normal anatomical or tissue-specific limitations. These changes often coincide with a dedifferentiated phenotype that may be a distinct consequence of the underlying genetic defects. At this early stage, potentially immunogenic tumor-associated shared "self" antigens [97] and truly foreign neoantigens [97, 98] first appear as epitopes found within proteins derived from mutated or dysregulated genes. Thus, in order to become established, grow, and progress, CNS cancers must evade the immune system even at this early stage.

3.1.2. Important Events in Tumorigenesis. Tumorigenesis is the process by which nascent oncogenic cell clusters transform into a viable tissue environment with a secure vascular supply and robust stromal elements capable of supporting the rapid and sustained tumor tissue growth. This transformation involves a complex series of events. Firstly, stromal elements must be recruited and developed into a subtumoral compartment that serves as a scaffold and provides crucial growth factors leading to angiogenesis and tumor tissue maintenance [56–58, 67–69, 71, 99, 100]. This stroma must be capable of supporting and promoting a dominant local immune suppression that leads ultimately to crucial tumor tolerance [11, 43, 62, 87, 101–106]. Furthermore, this tolerance, and the stroma that supports it, is characterized by a paradoxical inflammatory milieu that consists of chronic, low-grade, specialized inflammation, which we speculate may drive a characteristic "tissue remodeling" program that is normally meant for sterile wound healing, and which is actively suppressive for *de novo* T-cell responses within that milieu [59, 67–69, 71, 74, 88, 99, 107–110]. Thus, tumor survival is dependent upon these closely related and complimentary mechanisms: stromal formation and angiogenesis, immune suppression leading to the establishment of tolerance, and maintenance of both of these by a paradoxical inflammatory program usually reserved for sterile wound healing (Figure 1).

3.1.3. Stromal Formation and Angiogenesis. Development of vascular access for nutrient delivery is essential for early cancer cell clusters to develop into a tumor capable of further growth. Thus, the developing tumor must attract primitive stromal elements that can provide the foundation for tumor vascularization. This requirement defines the tumor microenvironment as an inflammatory tissue environment where chemokines [67, 68, 71, 88, 100, 108], cytokines [59, 67–69, 71, 74], and various growth factors [67, 68, 74, 87, 107, 109, 110] provide critical signals for migrating stromal elements—myeloid cells, vascular and lymphatic endothelial cells, pericytes, fibrocytes, fibroblasts, fibroblastic reticular cells, and so forth—to take up residence and functionally support tumorigenesis in the periphery. Once present, many of these stromal cell types, such as vascular [74] and lymphatic [75] endothelial cells, can engage in proliferation and can, themselves, secrete chemokines, cytokines, and growth factors to support the everincreasing stromal needs of the growing tumor [74, 75]. Thus, the sterile inflammation

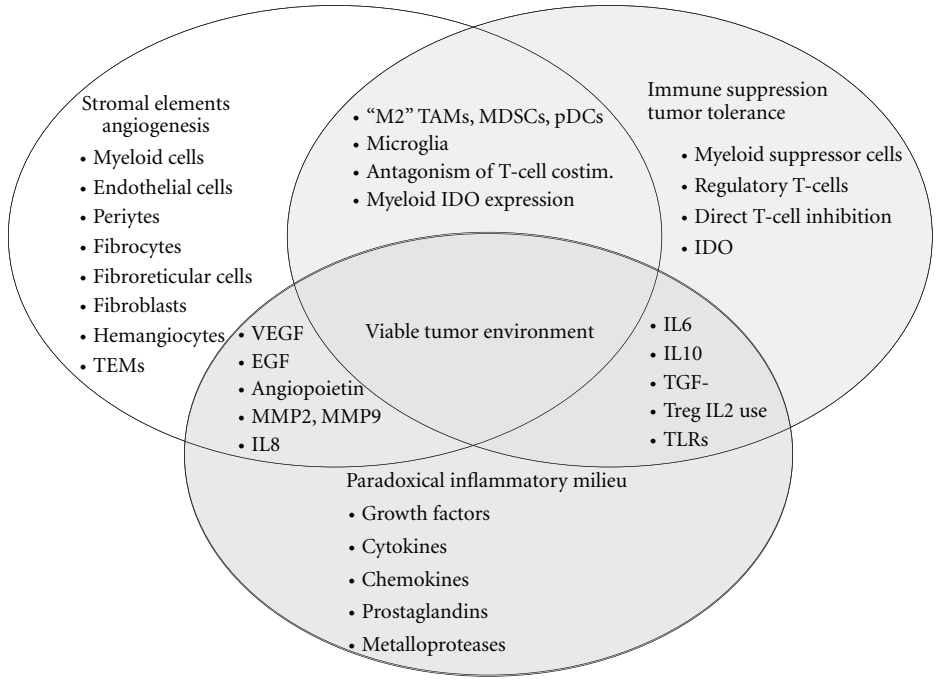

Figure 1: Viable tumor environment. Tumor survival is dependent upon an exquisite interplay between the critical functions of stromal development and angiogenesis, local immune suppression and tumor tolerance, and paradoxical inflammation. TEMs: TIE-2 expressing monocytes; "M2" TAMs: tolerogenic tumor-associated macrophages; MDSCs: myeloid-derived suppressor cells; pDCs: plasmacytoid dendritic cells; co-stim.: co-stimulation; IDO: indoleamine 2,3-dioxygenase; VEGF: vascular endothelial growth factor; EGF: epidermal growth factor; MMP: matrix metaloprotease; IL: interleukin; TGF-β: transforming growth factor-beta; TLRs: toll-like receptors.

of the tumor microenvironment recruits a complex stromal network to promote tumor growth and tissue remodeling as necessary. In fact, tumor tissue remodeling is necessary for the initiation of angiogenesis and occurs in a dynamic fashion [74], with a downregulation of antiangiogenic secreted proteases, such as ADAMTS-8 in brain tumors [107], and increased secretion of proangiogenic matrix metalproteases (MMPs), such as MMP2 and MMP9 [58, 67].

Many tumors, including gliomas, are capable of secreting other growth factors, such as vascular endothelial growth factor (VEGF) [72, 91], TGF-β [56–59], and progranulin [109, 110]. However, the intricate and crucial process of angiogenesis is mediated largely by CNS tumor infiltrating macrophages [67, 68] and microglia [56–58]. In fact, several tumor-associated macrophage subsets directly promote angiogenesis. Tumor-associated macrophages of the tolerogenic "M2" phenotype drive angiogenesis by secreting VEGF, MMP9, epidermal growth factor (EGF), and interleukin-8 (IL8) [67, 71]. Although their exact role in angiogenesis remains to be elucidated, Tie-2 expressing monocytes (TEMs) inhabit perivascular areas where Tie-2 serves as the receptor for angiopoietins [67, 68, 71]. Other, more heterogeneous myeloid populations involved in angiogenesis include hemangiocytes described as expressing CXCR4, VEGF receptor-1, Tie-2, Sca-1, and CD117 [67, 68] and myeloid-derived suppressor cells (MDSCs) which express CD11b, Gr1, and CXCR4 [67–69]. In CNS tumors, resident brain microglial cells migrate into the developing tumor in response to the same chemotactic signals that attract the myeloid subsets [39]. Although not as well studied, it is clear that microglia also contribute to angiogenesis by secreting VEGF, EGF, TGF-β, and MMP9 [57, 72].

3.2. Functional versus Anatomical CNS Privilege. As noted above, inflammatory responses in the CNS are more tightly regulated than other sites [29, 34, 35, 38, 86]. Historically, it has been assumed that much of this reduction in immune responses was a passive anatomical phenomenon, resulting from the lack of effective antigen-presenting cells and lymphatic drainage, combined with anatomic exclusion of circulating lymphocytes by the blood-brain barrier. Given this, it was natural to assume that CNS tumors partook of a similar, anatomically-based protection due to their "privileged" location [28, 111]. Such mechanisms doubtlessly play a role, but a new paradigm is also emerging, in which CNS tumors also exploit mechanisms of active immune suppression—both natural suppressive mechanisms that exist within the CNS and pathologic immunosuppressive mechanisms induced by the tumor. Together, these mechanisms allow tumors to actively protect themselves from immune clearance [28, 42, 111]. The need for active immune suppression becomes logical when we remember that the presence of the tumor itself often disrupts many of the passive anatomic barriers in the CNS, for example, by altering the blood-brain barrier in the tumor vasculature, enhancing leukocyte trafficking, creating chronic inflammation, and introducing new populations of antigen-presenting cells inside the tumor. Thus, tumors in the CNS are not "invisible" to the immune system, and

tumors must actively suppress immune responses against themselves in order to survive. The importance of understanding these active mechanisms of suppression lies in the fact that active mechanisms represent attractive therapeutic targets if they can be disrupted.

3.3. Local Immune Suppression and Establishment of Tumor Tolerance. As tumor size increases, tumor cell turnover also increases—and so does the volume of tumor-derived antigens. Many of the tumor-associated "shared-self" antigens could potentially be recognized by the immune system, because they may be excluded from central tolerance by virtue of their cellular, anatomical, or developmental expression patterns [97]. In the case of authentic tumor-specific neoantigens, which are derived from the protein products of mutated genes [97, 98], the immune system by definition has never acquired central tolerance. Despite this, however, the immune system behaves as if it were tolerant to tumor-derived antigens, whether shared "self" or neoantigens. One possible hypothesis to explain the lack of immune response in the presence of large amounts of these potential immunogens is that, analogous to the processes important in maintaining adaptive immune tolerance to normal tissues undergoing rapid cell turnover [112], antigens may be processed locally in a manner that avoids systemic immune activation. It appears that the type of APC that processes these antigens is critically important to the outcome—tolerance versus stimulation [113]—but the specific molecular mechanisms by which tolerance is created remain unclear. Nevertheless, the result can be dramatic: in one murine spontaneous-tumor model in which every tumor cell carries a potently immunogenic xenoantigen, the immune system still invariably becomes tolerant to the xenoantigen unless the host is vaccinated against the xenoantigen *prior* to tumorigenesis [102].

3.3.1. General Issues Regarding Suppression of Antitumor Immunity. Autochthonous peripheral tumor models suggest that tumor-specific tolerance may become established very early in tumorigenesis, as observed in mouse models of pancreatic ductal adenocarcinoma [101], 4T1 mammary tumors [43], B16F10 melanoma [43, 45], and AB1 mesothelioma [43]. This observation can be explained conceptually, in part, by the cancer immunoediting hypothesis in which an initially effective antitumor response "edits" the tumor cell repertoire by removing any cells that are immunogenic [62, 104]. Thus, in this model, the early interactions between immune cells and tumor cells actively select for later immune suppression by favoring tumor cells capable of escaping immune clearance.

Some of these escape mechanisms are passive. Passive tumor escape mechanisms were the first to be discovered and explored, and these include the emergence of tumor cell antigen-loss variants, downregulation of MHC-I expression, impairment of antigen processing or MHC binding in tumor cells, and suboptimal costimulatory molecule expression on tumor cells [62]. However, more recently, a variety of active immune suppressive mechanisms have been identified, which lead to dominant and profound tumor-induced tolerance (Table 1). These active mechanisms include secretion of soluble immune-modulating factors by tumor cells themselves, direct suppression of lymphocyte activation or effector function, and recruitment of myeloid or lymphoid suppressor cells. Immunosuppressive cytokines and growth factors known to be secreted directly by tumor cells include IL6 [114], IL10 [60], TGF-β [56, 58, 59, 62, 115], and VEGF [62]. These soluble mediators may directly inhibit T cell activation, and this effect may be augmented by contact-mediated antagonism of T cell costimulatory pathways through ligation of cytotoxic T lymphocyte antigen-4 (CTLA-4) [11, 62] or the programmed death-1 (PD-1) receptor on T cells [11, 46, 62]. In fact, one of the PD-1 ligands, PD-L1, is upregulated by gliomas when the *PTEN* tumor suppressor gene is defective [66].

Tumors and their stromal components are known to actively recruit regulatory immune cell subsets [87, 88, 101, 106, 116], especially regulatory T cells (Tregs) [43, 71, 88, 98, 99, 105, 116]. Tregs exert direct suppressive effects upon CD4 T cells and CD8 T cells via secretion of suppressive cytokines (IL10 and TGF-β); consumption of IL2 in the local microenvironment (which deprives effector T cells of this critical growth factor); contact-mediated inactivation of antigen-presenting cells; induction of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) [7, 43, 71], which is discussed below. In addition, activated Tregs within the tumor microenvironment can polarize tumor-associated macrophages toward the "M2" suppressive phenotype [71]. It is clear that Tregs play an important role in many tumors, although the degree to which different tumors depend on Tregs probably varies with context.

Despite active suppression of adaptive immune responses, most tumors appear to have an inflammatory milieu resembling a state of chronic sterile wound healing [67, 68, 99, 117, 118]. This characteristic tumor-associated inflammation is critical for maintenance of stromal integrity [99], promotion of angiogenesis [74], and continued tumor tolerance [88, 99, 108]. While a wide variety of stromal elements contribute to the formation of this specialized environment, tumor cells [99, 118] and tumor-associated macrophages [67, 68, 74, 100, 117] secrete many of the key inflammatory mediators, including the growth factors, cytokines, chemokines, prostaglandins, and metaloproteases described above. Elaboration of these crucial factors may be driven by transcriptional activation caused by oncogenic mutations [119], toll-like receptor (TLR) signal transduction [99, 118], and/or cytokine- or growth factor-mediated signaling [74, 99]. Particularly important to the immune tolerogenic properties of the tumor are the effects of TGF-β secretion [59], including suppression of T-cell adaptive and natural killer (NK) cell innate antitumor responses, recruitment of suppressive myeloid cell subsets such as suppressive dendritic cells, TAMs, and MDSCs, and recruitment of regulatory T cell activity [7, 12, 59, 73]. As noted above, vascular [120] and lymphatic [75] endothelium may contribute to this inflammatory milieu with growth factors and chemokines, and it is widely appreciated that tolerogenic "M2" phenotype TAMs support angiogenesis by secreting VEGF, EGF, MMP9, and so forth [67, 71].

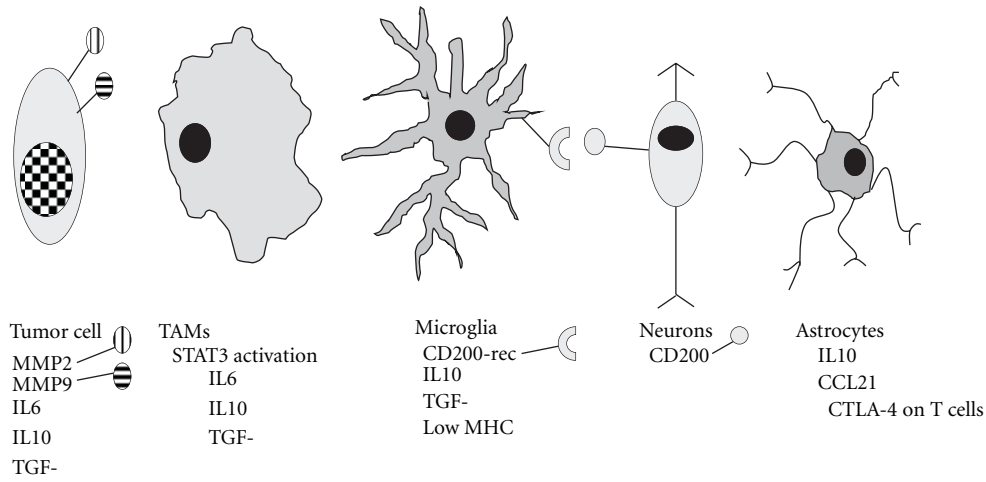

Figure 2: Tumor cells and stromal elements with immune suppressive functions. CNS tumor cells, especially glioma cells, may develop the ability to secrete cytokines including IL6, IL10, and TGF-β and can take advantage of membrane integrin-bound metaloproteases (MMP2 and MMP9) to facilitate motility and invasiveness. Tumor-associated macrophages (TAMs) bind IL6 and IL10 via their respective receptors, leading to phosphorylation and activation of STAT3, a transcription factor that upregulates TAM IL6, IL10, and TGF-β production and secretion. Ligation of the CD200 receptor on microglia by the ligand found on parenchymal neurons downregulates inflammatory cytokine and nitric oxide production by microglial cells. Microglial cells also have low expression of MHC-II and secrete IL10 and TGF-β. Astrocytes excrete IL10, and also CCL21, thus recruiting activated T cells which are then educated to upregulate CTLA-4 to antagonize costimulatory signals. IL10 promotes CNS tumor growth and migration, whereas TGF-β is an important regulator of tumorigenesis, angiogenesis, and tumor cell motility and invasiveness.

3.3.2. Infiltrating Tumor-Associated Macrophages and Microglia Maintain the Stromal Microenvironment and Suppress T-Cell Responses in CNS Malignancy. Tumor-associated macrophages (TAMs) and microglia are important for glioma tumor survival (Figure 2), as shown by the fact that ablation of these cells reduces tumor growth and improves survival in a murine syngeneic orthotopic glioma model [83]. TAMs process large volumes of dead and dying tumor cells without inciting adaptive immune responses, despite an apparently activated phenotype [67, 68, 71]. Tumor cell turnover is rapid in most solid tumors, and often the tumor core is devitalized as a result of central vascular insufficiency. This immense flux of cellular debris must be disposed of; a task that is largely borne by the tumor associated macrophages (but not, in the case of CNS tumors, by microglia) [39, 83]. This role for macrophages in tumors is reminiscent of other tolerogenic macrophage populations, for example, marginal zone macrophages which clear large amounts of apoptotic debris from the splenic circulation daily; tingible-body macrophages in germinal centers; Kupffer cells which process antigens from the portal circulation [112]. In none of these cases do macrophages provoke a pathological immune response to antigens from the dying cells that they ingest [112].

The classic trigger for inflammation is infection, in which activated APCs drive robust lymphocyte responses leading to pathogen clearance (albeit at the expense of local bystander tissue damage). However, as described above, certain inflammatory mediators are also critical to tumor establishment, growth, progression, and metastasis [68, 83, 117, 121, 122]. These occur in relatively tolerogenic tissue environments where adaptive immune responses against tumor-derived antigens have been blunted [100, 123]. This illustrates the point that the ultimate effects of inflammatory mediators depend not only on the character of the inflammation itself (e.g., sterile wound-healing tissue-remodeling type versus microbial pathogenic immune stimulation type) [99, 117, 118], but also upon the context in which it occurs (e.g., the actively immunosuppressive environment of tumors versus the stimulatory environment of infected tissue) [31, 35, 100, 123].

Outside the CNS, it is known that stromal elements in tumors can contribute to tumor tolerance. In addition to the role of macrophages described above, mesenchymal cells and fibrocytes that express fibroblast activation protein can drive tumor tolerance independently of TAMs [87]. In addition, both stromal cancer-associated fibroblasts within the tumor and mesenchymal fibroblastic reticular cells and lymphatic endothelial cells in the tumor-draining lymph nodes are capable of secreting CCL21 [108, 124], which has been shown to attract CCR7-expressing tolerogenic cell populations (including Tregs and IDO-expressing cells) [88]. In the specialized environment of the CNS, these and other stromal cell subsets are normally excluded, and astrocytes perform many of the comparable stromal functions. As mentioned above, CNS tumors often disrupt the normal architecture of the brain, so some of the stroma in brain tumors may be ectopic, and resemble stroma in peripheral locations. However, astrocytes also have the ability to suppress T cell responses—both directly via upregulation of CTLA-4 expression [40] and by recruitment of regulatory

T cells [125]. Also, CCL21 is secreted by glioma cells and tumor stromal cells and has been shown to directly promote glioma cell growth *in vitro* [83]. Astrocytes may therefore play a role in stromal-mediated tumor tolerance in the CNS.

Glioma-derived tumor cells are capable of directly secreting immunosuppressive cytokines [35, 39], including IL6 [114], IL10 [60], and TGF-β [56, 115], and microglia and astrocytes have also been documented as sources of cytokines [57, 60]. Serum IL10 levels are elevated in patients with high-grade glioma, and IL10 enhances glioma cell growth and migration *in vitro* [60]. Although high levels of TGF-β can inhibit glioma cell growth *in vitro* [115], *in vivo* TGF-β plays a role in glioma tumorigenesis, angiogenesis, cellular motility, and invasiveness [56, 57, 115]. This characteristic enhancement of invasive potential is mediated by increased secretion and integrin-mediated glioma cell surface binding of MMP2 and MMP9 [56, 58]. Cytokines not only affect the tumor cells, but they also affect the neighboring tumor-associated macrophages as well. Cytokines such as IL6 and IL10 bind their respective receptors on the cell surface of TAMs, leading to phosphorylation and dimerization of signal transducer and activator of transcription-3 (STAT3). An autocrine loop is thus established, whereby additional IL6 and IL10 are produced as a result of their own signal transduction by TAMs, which also begin to secrete TGF-β as a result of phospho-STAT3 transcriptional activation [13, 61, 121]. Thus, a mutually reinforcing interplay may exist between stromal cell- and glioma-derived immune suppressive cytokines, the stromal cells (macrophages, astrocytes, and microglia), and the glioma cells themselves whereby tumor-related growth, invasiveness, and immunosuppression are regulated.

3.3.3. Tumor Tolerance Mediated by Indoleamine 2,3-Dioxygenase. Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme, involved in tryptophan catabolism, which is expressed by several murine and human APC subsets that engage in suppression of T-cell responses [14–24, 45, 47–49, 126–129]. IDO enzymatic activity degrades tryptophan via oxidative cleavage of the pyrrole ring, which results in production of kynurenine as well as other downstream metabolic products, including picolinic acid, quinolinic acid, and 3-hydroxyanthranilic acid [14, 130, 131]. IDO expression by specialized plasmacytoid dendritic cells in tumor-draining lymph nodes directly suppresses local tumor-specific T cell responses in the periphery and promotes activation of regulatory T cells [16, 20–22, 47]. Direct T cell suppression via IDO-expressing APCs occurs through activation of the general control nonrepressed-2 (GCN2) kinase pathway in T cells which are attempting to activate in the context of insufficient tryptophan stores [132]. GCN2 kinase is part of an integrated stress response pathway that senses uncharged tRNA and leads to abortive T cell activation. Recent work has also implicated the downstream tryptophan catabolites themselves in suppressing T cell responses by tumor-infiltrating lymphocytes and in experimental models of autoimmune encephalitis [130, 131].

In a number of peripheral tumor models, IDO appears to function as a pivotal regulator of tolerance in the tumor-draining lymph node. IDO is expressed by specialized dendritic cells and other myeloid cells that potently suppress T cell responses [16, 18, 133]. Furthermore, IDO expression by dendritic cells in tumor-draining lymph node is necessary for certain forms of tumor-induced tolerance, a phenomenon which occurs in part via recruitment and induction of existing and new regulatory T cells [48]. Dendritic cells have been shown to drive T cell tolerance via the IDO pathway in both human [16] and murine [18] systems. Studies in mouse melanoma have shown that IDO expression by dendritic cells in draining lymph nodes suppresses CD8 T cell responses and leads to systemic tumor tolerance within just a few days [45, 48]. IDO can be induced by type I and type II interferons, by activated Tregs via CTLA-4 induced ligation of dendritic cell B7 molecules [22] and by STAT3-dependent mechanisms [134, 135].

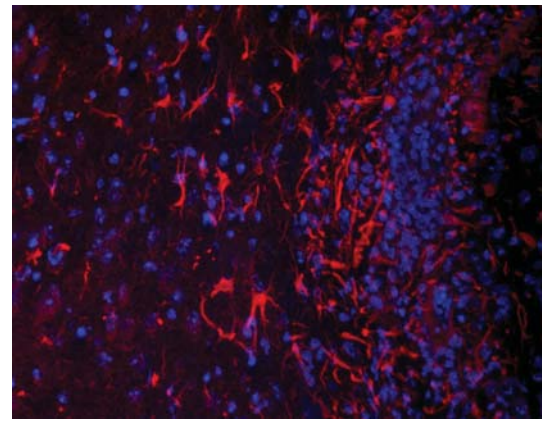

Figure 3: IDO-expressing astrocytes at the margin of a murine intracerebral GL261 glioma. IDO (red); nuclear counterstain (blue).

Most of the preceding studies focused on dendritic cells, which are notably lacking in CNS tumors. Less is known about the role of IDO in TAMs and tumor-associated glial and microglial cells. Several lines of evidence suggest that IDO may play a role in suppressing CNS tumor-specific immune responses. Using immunohistochemistry, Uyttenhove demonstrated widespread IDO expression in nine of ten human glioblastoma biopsies. [50]. Human glioma cells upregulate IDO expression and enzymatic activity in response to Interferon-γ (IFN-γ) treatment *in vitro* [136]. IDO expression also can be induced by IFNγ in astrocytes, microglia, and perivascular macrophages both *in vitro* and *in vivo* as the result of CNS inflammation [41]. Furthermore, intense IDO expression is seen in astrocytes within a reactive gliosis at the margin of orthotopic murine glioma tumors (Figure 3), and IDO activity has been documented in TAMs from a rat orthotopic glioblastoma model using an immunohistochemical method to stain tissue for quinolinic acid, a downstream tryptophan metabolite [137]. Thus, IDO is expressed by many CNS tumors and their associated stroma, but mechanistic studies are needed to elucidate the immunologic role of this IDO expression.

3.4. Leukocyte Tra cking and Maintenance of Quiescent Vascular Endothelium within CNS Tumors. Leukocyte entry

into the CNS is tightly regulated and appears to occur only by transmigration across the endothelium of post-capillary venules in the choroid plexus, meninges, and CNS parenchyma [38]. Thus, the most direct route for activated T cells to reach target tumor cells is transmigration across vascular endothelium within the tumor itself. This process is initiated via interactions between vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on endothelial cells and $\alpha_4\beta_1$-integrin and leukocyte function-associated antigen-1 (LFA-1), respectively, on T cells [29, 38, 84, 85]. In peripheral tumors, routine leukocyte margination within the tumor vasculature is hampered by diminished adhesion to the vessel walls, and this is thought to be the result of decreased endothelial adhesion molecule expression [76]. Chemo-attractant mediators may play an important role in activating the endothelial compartment so that it can support leukocyte binding for transmigration. Furthermore, leukocytes bearing chemokine receptors, including CCR1, CCR2, CCR7, and CXCR3, have been described in various models of CNS inflammatory disease and malignancy [29, 38]. Thus, the tumor may further shield itself from immune clearance by controlling the nature and quantity of cytokine and chemokine secretion by stromal elements, including antigen-presenting cells.

While endothelial quiescence is an important mechanism whereby leukocyte trafficking into CNS tumors is minimized, CNS tumor vasculature is nonetheless profoundly aberrant, with significant downregulation of endothelial adhesion molecules [38, 76]. In addition, there is considerable crosstalk between the stromal and endothelial compartments, which is only complicated by contributions of the tumor cells and marginated immune cells to the inflammatory milieu. Furthermore, effective angiogenesis must occur for tumor survival, and this process is mediated both via VEGF secretion by tumor and stromal cells [38, 39] and by secretion of angiopoietins which sustain and augment the vasculogenic process by binding the receptor tyrosine kinase Tie-2, expressed by endothelial lineage cells [67, 68, 71, 77–79]. In an intriguing departure from lineage specificity, glioma-derived stem cells can engage in vasculogenic mimicry, giving rise to aberrant intratumoral vascular endothelium [138–143], which has been shown to be both radioresistant [140] and chemoresistant [141].

4. Therapeutic Strategies to Break Immune Privilege

4.1. Vaccination against Brain Tumor-Specific Antigens. In the face of the profound tumor-induced tolerance driven by the mechanisms detailed above, it is not surprising that attempts to develop vaccination-based immunotherapy have been met with difficulty. In murine brain-tumor models, vaccines can create early signs of immune responsiveness (microglial upregulation of MHC, reactive gliosis, and lymphocytic infiltration), but fail to produce tumor rejection [80]. More intensive immunotherapy, combining peptide-pulsed dendritic cell vaccination with tumor-specific T cell adoptive transfer, showed that tumor-specific T cells do migrate into the brain tumor resulting in smaller tumors with prolonged survival [144]. However, these regimens were demanding, requiring sublethal irradiation prior to T cell transfer and dendritic cell vaccine as well as IL2 cytokine therapy afterwards.

Clinically, several very promising vaccines have been developed to target antigens on brain tumors [28]. Unfortunately, vaccination strategies against human glioblastoma have proven disappointing when used as single-agent therapy. Despite generating apparently robust circulating T cell responses, vaccines alone do not eradicate the brain tumors against which they are directed, nor do they provide gains in survival [81]. More encouragingly, however, when vaccines against brain tumors are used in conjunction with chemotherapy, the combination strategy has shown improvements in median progression-free and overall survival, although the emergence of antigen loss variants ultimately lead to tumor progression in a large majority of cases [82]. Thus, the promise of targeted vaccination strategies for treatment of CNS tumor patients remains an exciting area of research, but lacks sufficient efficacy to qualify as a standard therapy. For this reason, it is critical to understand the molecular mechanisms that contribute to tumor-related immune privilege in the CNS—especially those mechanisms that may be targeted by available therapeutic agents.

4.2. Pharmacological Blockade of Indoleamine 2,3-Dioxygenase. In various mouse tumor models, pharmacological inhibition of IDO can transiently break IDO-mediated tolerance and can improve the effectiveness of a number of chemotherapeutic agents, in an immune-mediated fashion [51, 52, 136]. A small molecule inhibitor of the IDO pathway (1-methyl-D-tryptophan, 1MT) is in Phase I and Phase II clinical trials for treatment of peripheral tumors in adult patients [145]. 1MT is not directly cytolytic to tumor cells [45, 52, 136, 145], but many chemotherapy agents are known to synergize with 1MT [52]. Recently, 1MT has been shown to reduce IDO activity in human-derived glioma cell preparations *in vitro* without diminishing the cytotoxicity of standard chemotherapeutic drugs, such as temozolomide [136]. However, no *in vivo* studies of 1MT have been reported, as yet, in preclinical brain-tumor models.

4.3. Antiangiogenesis Therapy. Despite the strong rationale behind developing antiangiogenic drug candidates [138], agents such as bevacizumab, a humanized monoclonal antibody that targets VEGF, have yielded mixed results [3, 146, 147]. Animal studies have shown anti-VEGF therapy to be effective at compromising glioblastoma perfusion by eliminating intratumoral vessels [146] via an apoptotic pathway [147]. However, intratumoral hypoxia appears to exert selection pressure upon glioma cells, increasing their invasive potential [146]. Furthermore, clinical trial data show conflicting results with significant extension of progression-free survival but no improvement in overall survival, relative to historical controls, in patients treated with bevacizumab and temozolomide [3]. These observations have raised the question of whether anti-angiogenic drugs may actually

compromise delivery of adjuvant chemotherapy to the tumor bed and thereby decrease effective glioma drug exposure.

4.4. Other Potential Strategies for Breaking Tolerance to CNS Tumors. Other agents that may be beneficial for brain tumor immunotherapy are also approved or in the pipeline. Contact-mediated antagonism of T cell costimulation by ligation of CTLA-4 [11, 62] or PD-1 has been shown to inhibit tumor-directed T cell responses [11, 46, 62]. PD-L1, one of the ligands for PD-1, can be expressed by glioma cancer cells as a protective mechanism [66]. Recently, ipilimumab, a monoclonal antibody that blocks signaling through CTLA-4, was approved by the Food and Drug Administration (FDA) for use in treating metastatic melanoma [63–65], and it has begun Phase III clinical trials for use in metastatic castration-resistant prostate cancer [64]. In addition, a monoclonal antibody that targets PD-1 signaling is in early-phase clinical trials for solid tumors, including prostate cancer. Although these drugs have yet to be tested for efficacy in CNS tumors, they represent promising avenues of immunotherapy that may be useful in targeting brain tumor tolerance in the future.

5. Conclusions

Malignant central nervous system tumors are resistant to standard radiation and chemotherapy following surgical extirpation. The specialized immunology of the CNS excludes or attenuates effective immune responses in malignancies. However, despite the complexity of this "CNS immune privilege", it is possible to recruit and activate lymphocytes and myeloid cells under certain conditions. Gaining a better understanding of CNS tumor-specific tolerogenic mechanisms is critically important to improving the survivability of this disease, which currently resists our most aggressive and multimodal therapeutic strategies.

Tumor-induced immune tolerance is robust, because successful tumors have been selected throughout their existence for their ability to evade the immune system. Even during the earliest stages of tumorigenesis, when high cell turnover and availability of tumor shared "self" antigens have the potential to awaken the otherwise quiescent immune system, CNS tolerance mechanisms must be intact for tumor survival. The specialized stroma of CNS tumors is likely to be critical to maintenance of immune suppression within their "sterile inflammatory" microenvironment. Infiltrating microglia, macrophages, and astrocytes make up this stromal milieu and maintain tumor tolerance through a variety of mechanisms, including secretion of immune suppressive cytokines and growth factors, suppression of local T cell responses, and recruitment of regulatory T cells. Vaccination strategies to recruit the immune system to drive tumor clearance must first overcome these tolerogenic mechanisms. Promising new therapies, such as IDO-inhibitor drugs and other checkpoint-blockade strategies, used with vaccines in multimodal combination chemoimmunotherapy regimens, may allow immunologic therapy of brain tumors to reach its full potential.

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Research Article

Combination Immunotherapy with 4-1BBL and CTLA-4 Blockade for the Treatment of Prostate Cancer

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Immune regulation has been shown to be involved in the progressive growth of some murine tumours. Interruption of immune regulatory pathways via activation of 4-1BB or cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) blockade appears to be a promising strategy for cancer immunotherapy. In this study, we examined the effectiveness of 4-1BBL-expressing tumor cell vaccine in combination with CTLA-4 blockade on rejection of murine prostate cancer RM-1. We found that the combination of both a vaccine consisting of 4-1BBL-expressing RM-1 cells and CTLA-4 blockade resulted in regression of RM-1 tumors and a significant increase in survival of the tumour cell recipients, compared to that of either treatment alone. The combined vaccination resulted in higher CTL against RM-1 cells and increased secretion of IFN-γ, TNF-α, and IL-2 in the mix-cultured supernatant. These results suggest that combining activation of 4-1BB and blockade of CTLA-4 may offer a new strategy for prostate cancer immunotherapy.

1. Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in old men and also the second leading cause of male cancer death in the western countries [1]. In addition, the incidence and mortality of carcinoma of prostate are increasing in China. Although radical prostatectomy and radiation therapy remain the optimal choice for localized stage of PCa, there is no effective treatment for patients who develop recurrences or develop into hormone-resistance prostate cancer (HRPC) or those who have metastatic disease at the time of diagnosis. Therefore, new therapeutic approaches to control or even eliminate residual tumor cells are definitely needed, providing an opportunity for immunotherapy [2].

It is well known that T-cell-mediated immune response plays a great important role in antitumor immunity. An effective T-cell response can attack tumor cells only after T cell receives two key signals from the peptide/MHC complexes and costimulatory signals (including B7-1/2, 4-1BBL, and CD40). Without costimulation, T-cells will undergo apoptosis or become anergic [3–5]. The fact that tumor cells are found to have low expression of costimulatory molecule may explain how tumor cells evade the immune surveillance. Consistent with this possibility, researchers demonstrated that conferring 4-1BBL expression to tumors of a variety of tissue origins was, in many cases, sufficient to promote tumor rejection by a $CD8^+$ T-cell-dependent mechanism [6, 7].

4-1BBL (CD137L), the counterreceptor for 4-1BB, is a member of the TNF (ligand) superfamily and serves as a secondary signal to activated T cells. 4-1BB signaling can induce cytokine production, expansion, and functional maturation of T cells, dendritic cells, NK cells, and monocytes [8, 9]. With regard to tumor biology, binding of 4-1BB has been demonstrated to prevent and even rescue anergic $CD8^+$ T cells in a number of tolerance-inducing models [10]. Also, 4-1BBL costimulation can retrieve CD28 expression in activated T cells [11]. A soluble 4-1BBL has also been shown to overcome immunological ignorance, allowing immunization with tumor-derived peptide to induce a protective CTL response [12].

CTLA-4, a close homolog of CD28, is upregulated on activated T cells and binds B7-1 and B7-2 with considerably greater avidity than CD28 results in the transduction of

an inhibitory signal and thereby functions as a negative regulator of T-cell activation in both $CD4^+$ and $CD8^+$ T cells [13]. When CTLA-4/B7 interactions are blocked by injection of anti-CTLA-4 monoclonal antibody during cancer vaccination, therapeutic T-cell immunity against even poorly immunogenic tumours such as B16 melanoma can be eliminated [14]. This effect is partly mediated by an increased expansion of antigen-specific CTL [15, 16]. It has been reported that blockade of CTLA-4/B7 interactions prevents induction of peripheral T-cell tolerance upon vaccination with peptides under tolerogenic conditions, suggesting that CTLA-4 might be actively involved in the induction of anergy [17].

In the present work, we investigated the effect of a vaccine combined with 4-1BBL-expressing tumor vaccine and CTLA-4 blockade on the survival of C57BL/6 mice transplanted subcutaneously with prostate cancer RM-1 cells. We found that 4-1BBL-expressing tumor vaccine in combination with CTLA-4 blockade was effective in reducing tumor incidence and increasing in survival of the tumour cell recipients.

2. Materials and Methods

2.1. Animals, Cell Lines, and Antibodies. Female C57BL/6 (H-2 K^b) mice, 6–8 weeks old, were obtained from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). Animals were maintained at the Central Animal Facility of Wuhan University according to standard guidelines, and experiments were conducted according to the guidelines of the China Council for Animal Care. All mice are killed by cervical dislocation in the experiment. RM-1, a murine prostate cancer cell line, was obtained from Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Anti-mouse CTLA-4 (clone 9H10) or hamster IgG isotype control was obtained from BioXCell; anti-4-1BBL was purchased from Santa Cruz.

2.2. Stable Transfection of RM-1 Cells with 4-1BBL Plasmid. The RM-1 cells were transfected with 2 μg of pCDNA3.1-4-1BBL or empty vector by the mediation of 6 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After 2 days of culture, the cells were reseeded into a 10 cm dish and cultured for another 2 days; complete RPMI-1640 medium containing 1000 μg/mL G418 (Sigma, St. Louis, MO) was added to the culture. After 20 days of selection, all nontransfected cells died, and discrete clones were visible in transfected cells. These clones were expanded in the presence of 200 μg/ml G418; positive cells expressing 4-1BBL or not were named RM-1/4-1BBL and RM-1/ pCDNA3.1.

2.3. Western Blot Analysis. To determine 4-1BBL expression, positive cells (5×10^6) were lysed and subjected to SDS-PAGE. Then protein was transferred to a nitrocellulose membrane (Amersham, USA). The transferred membrane was probed with polyclonal goat anti-4-1BBL antibody, followed by a horseradish peroxidase-conjugated anti-goat IgG secondary antibody (Santa Cruz, CA). Antibodies on membrane were visualized by chemiluminescence (Pierce, Rockford, IL). Western blot for β-actin was used as an internal sample.

2.4. Subcutaneous Challenge and Immunization. Mice were shaved on the back and challenged subcutaneously with 2×10^5 RM-1 cells in PBS. At the same day or later as indicated, parental and transduced cells were incubated with 100 μg/mL mitomycin C (MMC) for 1 hour as cancer vaccine, and treatment was initiated by injecting 10^6 cancer vaccine cells (in PBS) subcutaneously into the left flank and repeated 3 and 6 d later. Treatment with 9H10 or control hamster IgG was started simultaneously or 3 d later with similar results. Antibodies were delivered intraperitoneally at 100 mg in PBS, usually followed by two 50 mg injections every 3 d. Tumor growth was scored by measuring perpendicular diameters. Mice were killed when the tumors displayed severe ulceration or reached a size of 1000 mm^2.

2.5. Generation of CTL Cultures and CTL Assay. Spleens were harvested from mice rejecting RM-1 cells and restimulated *in vitro* with MMC-treated RM-1 cells, and recombinant human IL-2 was added to a final concentration of 50 IU/mL. After 7 d, cells were collected and purified by Ficoll-Histopaque (Sigma-Aldrich) gradient centrifugation and served as effector cells. Target cells (2.5×10^5 per well) were cocultured with effector cells (5×10^4 per well) at different E : T ratios in 96 round bottom plates. After a 48-hour incubation at 37°C, the amount of released lactate dehydrogenase was determined by using Cell Counting Kit-8 (Dojindo, Japan) assay according to the manufacturer's instructions. All determinations were carried out in triplicate and repeated three times. The percentage of specific cytotoxicity was calculated as [target control − (experimental − effectorcontrol)/target control] × 100%.

2.6. Enzyme-Linked Immunosorbent Assay. 24 h after target cell cocultured with effector cells, the supernatant was collected and tested for the presence of IFN-γ, TNF-α, and IL-2 by ELISA according to the manufacturer's instructions (Pharmingen).

2.7. Statistical Analysis. Data were presented as mean ± standard deviation. Statistical differences were considered to be significant at a Pvalue <0.05 as determined by an ANOVA or Student's t-test using SPSS13.0. Comparison among groups in the survival data was made using the log-rank test.

3. Results

3.1. Establishment of RM-1 Cells Expressing 4-1BBL. RM-1 cells were transfected with pCDNA3.1 and pCDNA3.1/4-1BBL, and the G418-resistant cells (RM-1/pCDNA3.1, RM-1/4-1BBL) were selected. Western blot analysis showed the

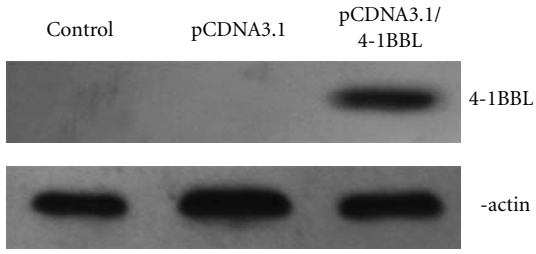

Figure 1: Total cell lysates were harvested, and presence of 4-1BBL protein was detected by anti-4-1BBL pAb. A specific band was identified in positive clone but not in RM-1 cells transfected with empty vector. β-actin was used as reference.

expression of 4-1BBL in parental and transduced cells, respectively (Figure 1).

3.2. CTLA-4 Blockade Together with 4-1BBL-Expressing Cellular Vaccines Causes Rejection of RM-1 Tumors. To determine the effect of 4-1BBL-expressing cellular vaccines combined with CTLA-4 blockade on tumor growth in vivo, parental RM-1, RM-1/pCDNA3.1, and pCDNA3.1/4-1BBL cells were injected into the flank of mice. Tumor growth in mice injected with pCDNA3.1/4-1BBL cells was slower than that in mice injected with parental RM-1 or RM-1/pCDNA3.1. Administration of anti-CTLA-4 antibody 9H10 delayed growth of RM-1 tumors, but control hamster IgG had no effect. However, the combination of 4-1BBL-expressing vaccine and CTLA-4 blockade induced more obvious effectiveness on RM-1 tumor growth than either treatment alone. 4/5 mice rejected RM-1 tumors after the combinated treatment (Figure 2).

3.3. CTLA-4 Blockade Together with 4-1BBL-Expressing Cellular Vaccines Increased CTL Activity and Production of Cytokines. To determine the immune function of 4-1BBL-expressing cellular vaccines combined with CTLA-4 blockade in vitro, CTL activity of splenocytes from immunized mice was evaluated. As shown in Figure 3, CTL activity of splenocytes from mice immunized with the combination of 4-1BBL-expressing vaccine and CTLA-4 blockade was dramatically higher than that from mice treated either alone. Vaccination with parental RM-1, RM-1/pCDNA3.1, and control hamster IgG had no effect on CTL activity. Also, the level of cytokines in supernatant cocultured cells was examined. The results showed that the levels of cytokines (IFN-γ, TNF-α, and IL-2) in supernatant from mice vaccinated with 4-1BBL-expressing vaccine and CTLA-4 blockade were much higher than that from mice immunized with either alone (Figure 4).

3.4. CTLA-4 Blockade Together with 4-1BBL-Expressing Cellular Vaccines Prolonged the Life Span of Mice Rechallenged Tumors. To determine the immune protection effect of 4-1BBL-expressing cellular vaccines combined with CTLA-4 blockade, mice were inoculated subcutaneously with lethal dose of parental RM-1 cells to monitor survival daily. The

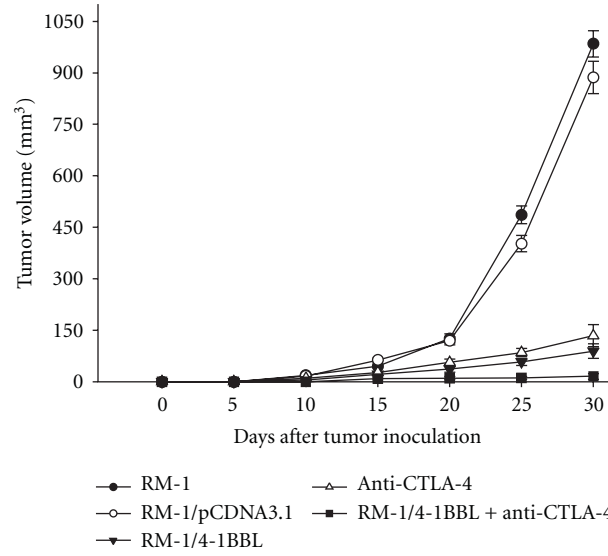

Figure 2: Antitumor immunity *in vivo*. Mice were challenged subcutaneously with RM-1 cells (2×10^5 cells/mouse), then 10^6 tumor vaccine cells (RM-1, RM-1/pCDNA3.1, RM-1/4-1BBL) were immunized at the same day, with 9H10 or control hamster IgG intraperitoneally every 3 d. The data are expressed as means ± SD of three replicates ($^*P < 0.05$).

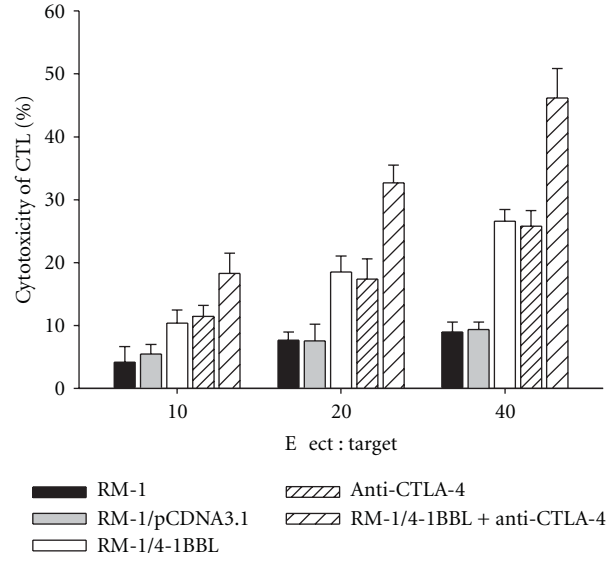

Figure 3: Cytotoxicity assay. Spleens were harvested from mice rejecting RM-1 cells and restimulated in vitro with MMC-treated RM-1 cells. 7 d later, cells were collected and purified by Ficoll-Histopaque gradient centrifugation as effector cells for detecting specific cytotoxicity against target cells. The data are expressed as means ± SD of three replicates ($^*P < 0.05$).

survival rate of mice immunized with both CTLA-4 blockade and 4-1BBL-expressing cellular vaccines was significantly higher than that of mice immunized with either alone (Figure 5).

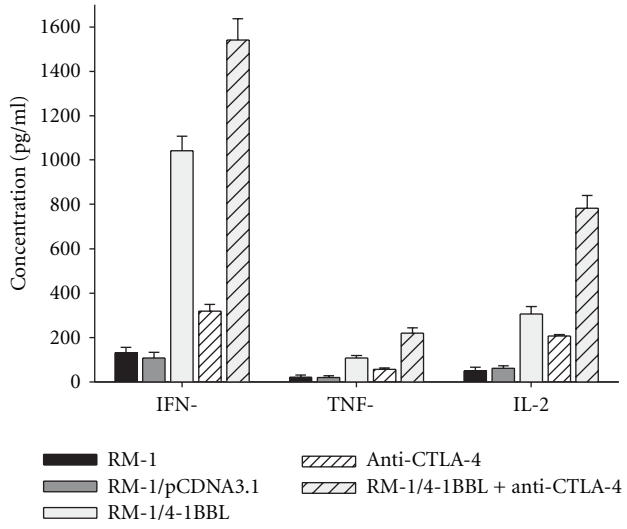

Figure 4: Cytokine ELISA. Spleens were harvested from mice rejecting RM-1 cells and restimulated in vitro with MMC-treated RM-1 cells. 7 d later, cells were collected and purified by Ficoll-Histopaque gradient centrifugation as effector cells coculturing with RM-1 cells. The The supernatant was collected and tested for the presence of IFN-γ, TNF-α and IL-2 by ELISA ($^*P < 0.05$).

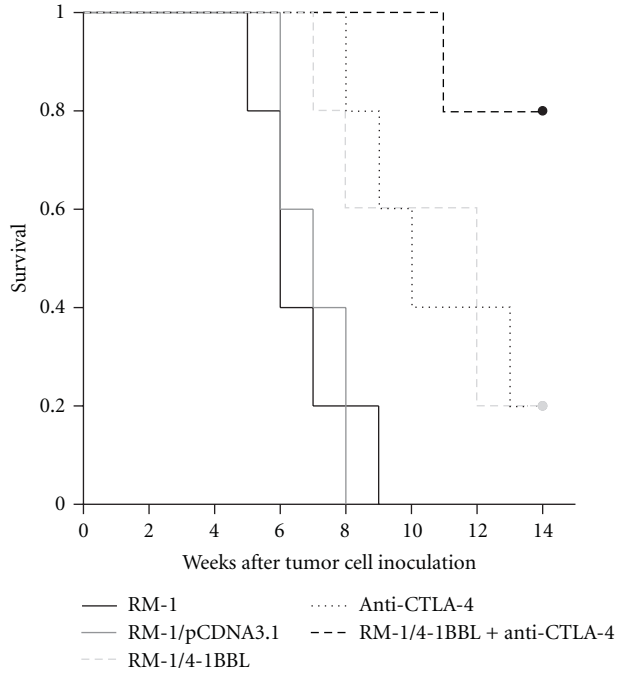

Figure 5: Kaplan-Meier survival curve of mice. According to the log-rank test, there were significant differences among five groups ($P < 0.05$). Compared with those of the other two groups, the survival rate of mice immunized with 4-1BBL-expressing cellular vaccines combined with CTLA-4 blockade was significant higher than those of mice immunized with either alone ($P < 0.05$).

4. Discussion

In the present study, we demonstrated that the preclinical effect of 4-1BBL-expressing cellular vaccines can be markedly improved by combining vaccination with treatment with anti-CTLA-4 mAb. The combination vaccine resulted in sustained tumor degradation in all the mice. Our treatment regimen holds great promise for a positive clinical effect in humans.

Actually, cancer occurrence and development has been demonstrated to be associated with escape from immune surveillance. The prostate cancer vaccine alone was unable to cause complete tumor regression, which could reflect either that the initial $CD8^+$ T-cell-mediated antitumor immune response simply is not potent enough to completely eliminate all the cancer cells or that the cancer cells have lost their immunogenicity. There might be several reasons for that including (1) low-level expression of the major MHC molecules, (2) absence of recognized tumor Ags, (3) poor costimulatory molecule expression, or (4) some kind of immunosuppression of the $CD8^+$ T-cell response such as TGF-β [18]. 4-1BB is an inducible member of the TNFR superfamily that has profound effects on T cells, including activation of both $CD4^+$ and $CD8^+$ T cells, enhanced expansion [19, 20], increased long-term survival [21, 22], and antiapoptosis of activation-induced $CD8^+$ T cells [23]. Costimulation through 4-1BB can also promote enhanced production of cytokines such as IL-2, IL-4, and IFN-γ [19, 24]. Regarding this, we sought to understand the effect on tumor-specific T cell responses of simultaneously actively driving proliferation and survival through activation of the costimulatory receptor 4-1BB, while at the same time eliminating a major brake on expansion via blocking the coinhibitory receptor CTLA-4.

In murine experiments, activation of 4-1BB with 4-1BB mAb can lead to rejection of many tumours [25]. Indeed, phase I and II clinical trials using anti-4-1BB therapy for advanced cancers are underway [26]. However, anti-4-1BB antibodies can cause severe immune system anomalies when given systemically [27]. Thus, in the present study, we use 4-1BBL-expressing cellular vaccines for treatment of prostate cancer. Our data indicate that the 4-1BBL-expressing cellular vaccine did slow tumor growth when initiated at the time of tumor implantation and resulted in regression of tumors in about 2/5 of the mice. Moreover, the combination of 4-1BBL-expressing cellular vaccine with CTLA-4 blockade induced rejection of all tumors injected at the same day. These results suggest that 4-1BBL-expressing cellular vaccines can be markedly improved by combining vaccination with treatment with anti-CTLA-4 mAb. In the study, we demonstrated the number of $CD8^+$ and $CD4^+$ T cells in RM-1 tumors in mice immunized with anti-CTLA-4 mAb and 4-1BBL-expressing cellular vaccines (data not shown), which suggest enhancement of cytotoxicity of TIL might be a way for the combination of vaccine to execute antitumor effect. The same results were observed in B16 melanoma by Kocak et al. [28] and Curran et al. [29].

Our results demonstrated that CTL activity of splenocytes and cytokine from mice immunized with 4-1BBL-expressing cellular vaccines and CTLA-4 blockade was dramatically increased compared with that from mice immunized with either alone. Vaccination with parental RM-1,

RM-1/pCDNA3.1, and control hamster IgG had no effect on CTL activity. The main effector cells performing CTL activity are $CD8^+$ T cells, while the main cells producing cytokines such as IL-2, IFN-γ, and TNF-α are $CD4^+$ Th1 cells. Th1 cells play a critical role in cellular immunity by their cytokines activating $CD8^+$ T cells. Long-term survival of mice immunized with 4-1BBL-expressing cellular vaccines and CTLA-4 blockade when rechallenged lethal dose of parental RM-1 cells indicated that the combination of vaccines executes antitumor immune response by activating $CD8^+$ and $CD4^+$ T cells.

The findings presented in this study have significant implications for immunotherapy in humans. Our results suggested that it is important to consider whether two treatments will act synergistically when developing an immunotherapeutic strategy. Moreover, they also suggest that CTLA-4 blockade may be a vital adjuvant for a 4-1BBL-expressing vaccine used to treat cancers.

5. Conclusion

In summary, we concluded that the combination of activation of 4-1BB and blockade of CTLA-4 has a higher potential antitumor effect and may offer a new strategy for prostate cancer immunotherapy.

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Clinical Study

Serum CEACAM1 Correlates with Disease Progression and Survival in Malignant Melanoma Patients

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The search for melanoma biomarkers is crucial, as the incidence of melanoma continues to rise. We have previously demonstrated that serum CEACAM1 (sCEACAM1) is secreted from melanoma cells and correlates with disease progression in metastatic melanoma patients. Here, we have used a different cohort of melanoma patients with regional or metastatic disease ($N = 49$), treated with autologous vaccination. By monitoring sCEACAM1 in serum samples obtained prior to and after vaccination, we show that sCEACAM1 correlates with disease state, overall survival, and S100B. The trend of change in sCEACAM1 following vaccination (increase/decrease) inversely correlates with overall survival. DTH skin test is used to evaluate patients' anti-melanoma immune response and to predict response to vaccination. Importantly, sCEACAM1 had a stronger prognostic value than that of DTH, and when sCEACAM1 decreased following treatment, this was the dominant predictor of increased survival. Collectively, our results point out the relevance of sCEACAM1 in monitoring melanoma patients.

1. Introduction

Malignant melanoma is a main cancer-related cause of death in people below 30. While its incidence continues to rise more rapidly than that of any other malignancy, until lately, therapy had shown only moderate success and caused numerous adverse effects [1–3]. A new hope for melanoma patients has emerged now from the development of a specific B-RAF inhibitor and the entry of immune checkpoint modulators to the clinic. In spite of this progress, the monitoring of melanoma patients still presents a clinical challenge as it heavily relies on history taking, physical examination, and wide imaging studies [4]. This, together with the fact that melanoma can remain dormant for long periods of time before relapsing [5], emphasizes the need for valid melanoma biomarkers. Currently, the two most widely used melanoma biomarkers are lactate dehydrogenase (LDH) and the calcium binding protein S100B [6–8]. Serum levels of S100B or LDH correlate with poor outcome and are associated with shorter disease-free and overall survival [9, 10]. Several studies showed the prognostic value of S100B and LDH in predicting successful therapeutic treatments for malignant melanoma patients [11–16]. Unfortunately, however, serum S100B and LDH are not specific for melanoma. Abnormal elevation of S100B accompanies liver and kidney injuries as well as inflammatory and infectious diseases [17], while elevated LDH is also observed in liver injury, cell damage, hemolysis, and so forth [18–20].

CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1) is a transmembrane multifunctional cell-cell adhesion molecule, belonging to CEACAM, a subdivision of the Ig Superfamily. Broadly expressed in human epithelial, endothelial, and hematopoietic cells, it regulates immune responses, neovascularization, and insulin clearance (reviewed in [21, 22]). Membranal CEACAM1 (mCEACAM1) expression is downregulated in some types

of cancer [23–26] and its reexpression by tumor cells inhibits *in vivo* tumor growth [27, 28], leading to the original definition of mCEACAM1 as a tumor suppressor. However, in several cancers, including malignant melanoma and non-small-cell lung cancer, mCEACAM1 is upregulated and its expression highly correlates with tumor progression, the development of metastasis, and poor survival [29–31]. Immunohistochemical analysis on superficial spreading melanoma, dysplastic nevi and benign nevi, showed that mCEACAM1 is stepwise elevated during the course of malignant melanoma progression [32]. Patient monitoring proved that its predictive value for metastasis formation and poor survival is superior to that of tumor thickness and independent of other factors, including ulceration, tumor thickness, and mitotic rate [29]. Mechanistic evidence regarding the role of mCEACAM1 in melanoma is scarce. *In vitro* studies have demonstrated that mCEACAM1 promotes melanoma cell migration and invasion [33] as well as protection from elimination by cytotoxic NK and T cells [34–36]. We have recently identified a soluble form of human CEACAM1 (sCEACAM1), which is produced and secreted from melanoma cells in a process that demands active protein synthesis and intact intracellular vesicular transport [37]. Monitoring of metastatic melanoma patients for serum levels of sCEACAM1 showed that patients with evidence of disease (WED) exhibit significantly higher serum sCEACAM1 levels as compared to patients with no clinical evidence of disease (NED) or with healthy volunteers. sCEACAM1 levels correlated with LDH, and most importantly, stratified the metastatic patients into two prognostic groups with different survival rates [37]. These results exhibit the prognostic value of sCEACAM1 for melanoma progression and survival.

In this study, we monitored melanoma patients with regional or metastatic disease, treated with autologous cell vaccination. Melanoma is unique among human cancers as it induces significant numbers of anti-tumor reactive lymphocytes during the natural course of tumor growth [38]. Vaccination with modified autologous melanoma cells given as a postsurgical adjuvant therapy is thought to elicit this naturally occurring immune response and to prolong disease-free period [39, 40]. Vaccination may be beneficial especially in selected patients who show successful anti-melanoma immune response, as reflected by the delayed-type-hypersensitivity (DTH) test (i.e., positive skin reaction to subcutaneous injection of unmodified autologous melanoma cells) [39, 40].

Here, we monitored 49 melanoma patients (AJCC stages III-IV) treated with autologous tumor vaccination in the years 1998–2010 and focused on sCEACAM1 evaluation. We found that sCEACAM1 correlates with disease state and is also likely to correlate with survival rate. Moreover, the change in sCEACAM1 over time (increase or decrease) correlated with overall survival and had a superior value over DTH skin response. In addition, post-vaccination sCEACAM1 correlated with S100B. These observations support the prognostic value of sCECACM1 and its potential role in monitoring of melanoma patients with regional or metastatic disease.

2. Patients and Methods

2.1. Patients. Patients with pathologically verified cutaneous MM in AJCC stages III-IV in the years 1998–2010 were included. Two patients with thick cutaneous melanomas AJCC stage IIB were included in this series, on a compassionate basis. Clinical characteristics of participants are detailed in Table 1. There were no exclusion criteria. All NED patients were treatment-naïve (were not treated before vaccination). WED patients were accrued on the condition that they had progressed following first-line treatment (DTIC, IL-2 or both). Patients' evaluation was done by CT scan of the whole body, performed within 28 days prior to treatment initiation. All melanoma patients gave written informed consent prior to their participation in this study. This study was approved by the Institutional Review Board of Hadassah Hebrew University Hospital, Jerusalem.

2.2. Vaccination. The protocol used for vaccine preparation and delivery was as previously described [40]. Both NED and WED patients were treated with the same protocol. Briefly, $10–25 \times 10^6$ autologous melanoma cells were subcutaneously injected in each dose of vaccine. On treatment day, the cells were thawed, washed, and irradiated to 170 Gy. Conjugation of melanoma cells with DNP (dinitrophenol) was performed by the method of Miller and Claman [41]. Bacille Calmete Guerin (BCG) was used as an adjuvant and mixed with tumor cells. DNP sensitization was induced by applying 0.1 mL of 2% DNP dissolved in acetone-corn oil (Sigma Aldrich) topically to the inner aspect of the arm. The first two vaccine doses were preceded by cyclophosphamide, 300 mg/m^2, given as an immunomodulatory dose. The vaccine was injected into 3 adjacent sites on the upper arm or thigh, avoiding limbs where lymph node dissection has been previously performed. An overall of eight doses of vaccine were administrated at intervals of 21–28 days.

2.3. Specimen Characteristics. Blood samples were obtained from patients before the first vaccine was administered (usually up to 2 months after surgery), and following the 5th or 8th vaccination, by venipuncture and standard handling procedures. 15 milliliters of blood were collected in citrate-containing tubes (BD Biosciences) and then centrifuged at 700 g for 10 minutes in room temperature to obtain sera. All serum samples were collected and divided into aliquots and frozen in -80°C until analysis. Anonymous samples (marked only with ID number) were linked only to clinical-pathological data.

2.4. CEACAM1 and S100B Evaluation by ELISA. sCEACAM1 serum levels were measured by the Sandwich ELISA protocol described in [37]. Soluble S100B in the serum was estimated by ELISA according to the manufacturer's instruction [42]. Half of the samples were analyzed in Hadassah Medical Center and the others in Sheba Medical Center. The results obtained from the two medical institutes showed some differences, probably due to variability in sample handling, freezing/thawing cycles, and batches of antibodies used. In

Table 1: Depiction of the clinical characteristics of 20 WED and 29 NED melanoma patients used in this study.

Total number of patients	49 (100%)		
Age (years) at treatment		*Sex, Female*	25 (51%)
<40	9 (18.4%)		
41–60	16 (32.7%)		
>61	24 (49%)		
Stage at presentation		*Time from first treatment to last follow up date*	
Stage II	2	0–12 months	12 (24.5%)
Stage III	32	13–24 months	13 (26.5%)
Stage IV	15	25–36 months	4 (8.2%)
		>37 months	20 (40.8%)
Stage at treatment of NED patients ($N = 29$)		*Stage at treatment of WED patients* ($N = 20$)	
Stage II	2 (6.9%)	Stage III (unresectable)	10 (50%)
Stage III (respectable)	22 (76%)	Stage IV: M1a	1 (5%)
Stage IV: M1b	2 (6.9%)	M1b	1 (5%)
M1c	3 (10.3%)	M1c	8 (40%)
LDH values of NED patients		*LDH values of WED patients*	
Time point 0:		Time point 0:	
normal	87% (20/23)	normal	78% (11/14)
above normal	13% (3/23)	above normal	21% (3/14)
Time point 1:		Time point 1:	
normal	78% (18/23)	normal	64% (7/11)
above normal	22% (5/23)	above normal	36% (4/11)

Table 2: sCEACAM1 correlates with S100B. ELISA measurements of posttreatment sCEACACM1 and S100B yielded values that were divided relative to median levels into "low" and "high." The correlations between the two resulted "low" subgroups, as well as between the two "high" subgroups, were tested and found to be significant ($P = 0.02$). Percentages in each cubical refer to sCEACACM1 (first row) or to S100B (second row).

	Low S100B $N = 25$	High S100B $N = 23$
Low CEACAM1 $N = 29$	$N = 19$	$N = 10$
	65.5% (19/29)	34.5% (10/29)
	76% (19/25)	43.5% (10/23)
High CEACAM1 $N = 19$	$N = 6$	$N = 13$
	31.6% (6/19)	68.4% (13/19)
	24% (6/25)	56.5% (13/23)

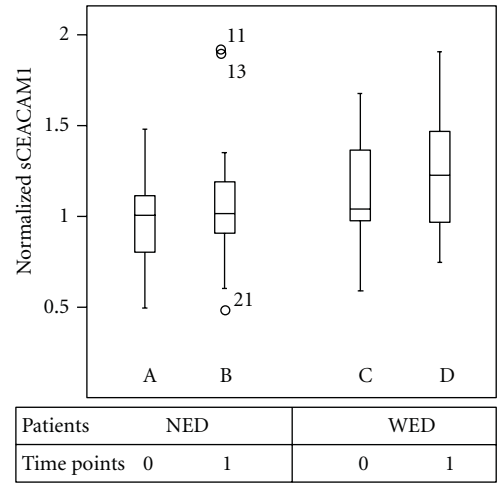


Figure 1: sCEACAM1 in WED patients increases over time and as compared with NED patients. sCEACAM1 was measured by ELISA in serum samples of 29 NED (A, B) and 20 WED (C, D) malignant melanoma patients, both before (time point 0) and following treatment (time point 1). Vertical lines indicate medians.

order to compensate for these differences, the two medians (one for each group of samples) were calculated, and each sCEACAM1 value was divided by the median of its group (Figures 1, 2 and Table 2). In this analysis, sCEACAM1 values which equal the median are represented by 1 and values high/lower than median by >1 or <1 values. Similar normalization was performed for S100B (Table 2). When analyzing ΔsCEACAM1 values (post-vaccinations 5th or 8th minus pre-1st vaccination levels, Figures 3-4), absolute rather than normalized sCEACAM1 values were used.

2.5. DTH Evaluation. Skin testing to evaluate delayed type-hypersensitivity to autologous melanoma cells was performed by intradermal injection of 1–3 × 10^6 unmodified melanoma cells irradiated at a dose of 170 Gy, as already described in [40]. We arbitrarily chose the value of 10 mm of

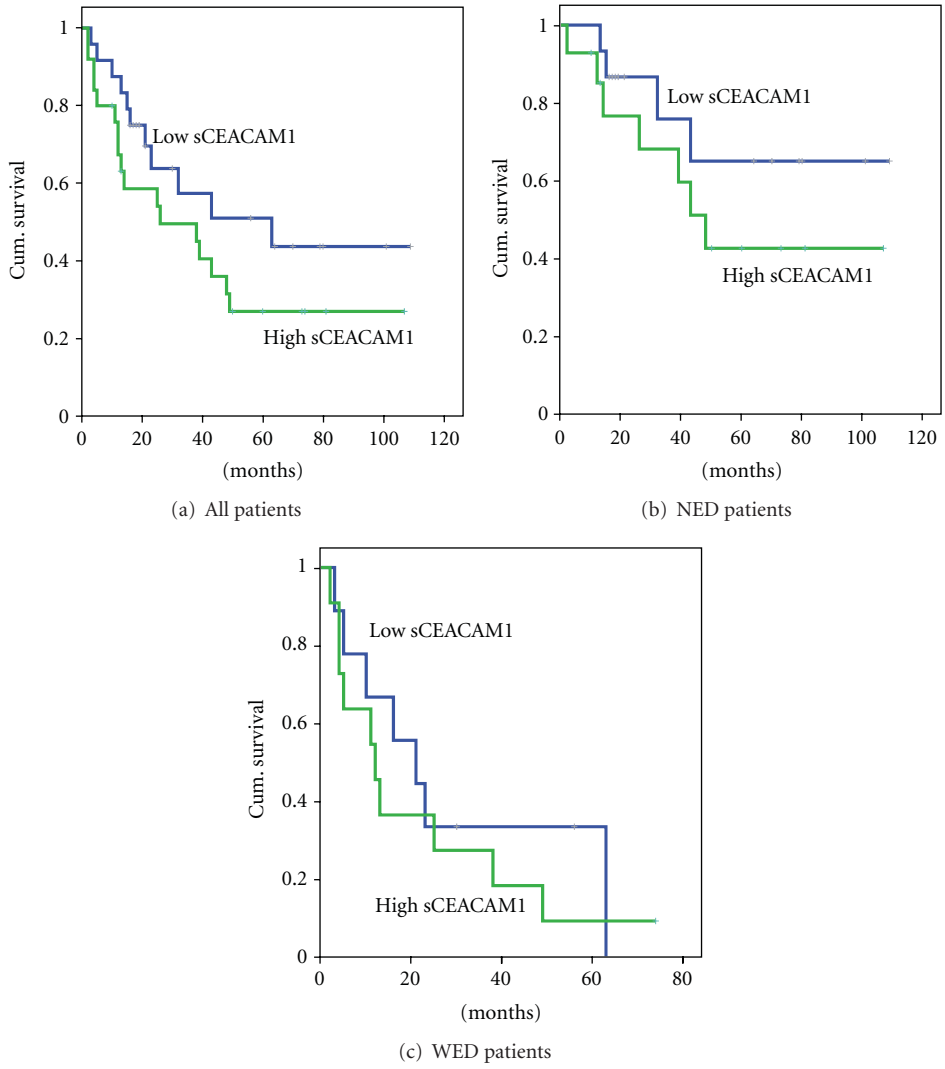


Figure 2: The correlations between sCEACAM1 and survival. The whole group of patients and NED exclusively or WED exclusively were divided into two groups ("low"/"high") according to pre-treatment sCEACAM1 median level, and the survival rate of each subgroup was analyzed by Kaplan-Meier analysis. Group sizes were as follows: $N = 24$ (low) and $N = 25$ (high) in (a); $N = 15$ (low) and $N = 14$ (high) in (b).

erythema to discriminate between negative DTH (<10 mm) and positive DTH (≥10 mm).

2.6. Study Design. This study was retrospective. No stratification or matching were used and patients (the great majority of them from AJCC stages III and IV) were selected in a random manner. Sample size ($N = 49$) matched previous similar studies (reviewed in [43]) and was sufficient for analysis of the results. Samples were obtained from June 1998 through October 2010. Median follow-up time was 23 months.

2.7. Statistical Analysis. The analysis was focused on the impact of sCEACAM1 on disease progression and survival. Overall survival was estimated by the Kaplan-Meier's method. Significance (P value) was calculated by Mantel-Cox regression.

3. Results

3.1. Soluble CEACAM1 Correlates with Disease State. Our study encompassed 49 melanoma patients, staged, based on AJCC 2002, as AJCC II ($N = 2$), III ($N = 32$), and IV ($N = 15$), that were treated with autologous vaccination (Table 1). The patients were categorized according to the clinical manifestation of disease into patients with no evidence of disease (NED; $N = 29$; 22/29 in AJCC III) and patients with active disease (WED; $N = 20$, 10/20 in AJCC III and 10/20 in AJCC IV). Accordingly, most patients exhibited normal LDH values (Table 1). It should be noted that autologous vaccination is beneficial for selected patients and

uncommonly yields objective tumor regressions [39, 40]. In our cohort of patients, it did not result in any tumor regression (Table 1). Measurement of serum CEACAM1 (sCEACAM1) in blood samples over time (i.e., before as compared to following vaccination; Figure 1, C as compared to D), revealed a 20% elevation of sCEACAM1 in the WED group and no elevation in the NED group (A as compared to B). Comparison of WED to NED patients demonstrated a 20% elevation in WED patients, both at basal time point (Figure 1, mean sCEACAM1 = 0.93 in a as compared to 1.11 in C; $P = 0.024$) and following vaccination (Figure 1, mean sCEACAM1 = 1.0 in B as compared to 1.19 in D; $P = 0.068$). These results are in line with our previous findings in a different cohort of melanoma patients and treatments, describing a significant elevation in sCEACAM1 in WED as compared to NED patients and healthy volunteers [37].

3.2. Soluble CEACAM1 Correlates with Survival in NED Patients. We next categorized the whole group of patients according to their basal (pre-treatment) sCEACAM1 values into "high" and "low" subgroups (see "*Methods*"). Analysis of overall survival rates using Kaplan-Meier plots revealed an inverse correlation between sCEACAM1 and survival (Figure 2). This correlation was evident though it did not reach statistical significance. While in the low-sCEACAM1 subgroup (Figure 2(a); black, $N = 24$), the mean overall survival rate was 62 months, it was only 44 months for high-sCEACAM1 patients (Figure 2(a); gray, $N = 25$) and 49 months for the whole population of patients. In order to rule out the possibility that these results stem from the fact that most low-sCEACAM1 patients (70.8%) were NED (i.e., patients whose expected survival is higher), the same analysis was performed for each of the patients groups separately. As can be seen in Figure 2(b), NED patients whose sCEACAM1 was low were likely to have a higher overall survival rate (black, 80.8 months, $N = 15$) as compared to sCEACAM1^{high} NED patients (Gray, 61 months, $N = 14$). In WED patients, pre-treatment sCEACAM1 had no prognostic value on survival rate (Figure 2(c)).

3.3. The Change in sCEACAM1 over Time Inversely Correlates with Survival. In order to test the correlation between sCEACAM1 and survival in the whole group, independently of patients' status as NED or WED, we calculated the change in sCEACAM1 after treatment for each of the 49 patients (ΔsCEACAM1). The sCEACAM1 levels before treatment served as the point of reference. Patients were divided into two groups according to the trend ("increased"/"decreased") of ΔsCEACAM1 and Kaplan-Meier analysis was performed for each of the groups (Figure 3). Remarkably, the 26 patients that exhibited a decrease in sCEACAM1 levels during followup were characterized by a mean overall survival rate of 63 months, whereas the 23 patients in which sCEACAM1 was increased had a mean survival rate of only 40 months ($P = 0.055$). The trend of change of sCEACAM1 thus positively correlated with survival.

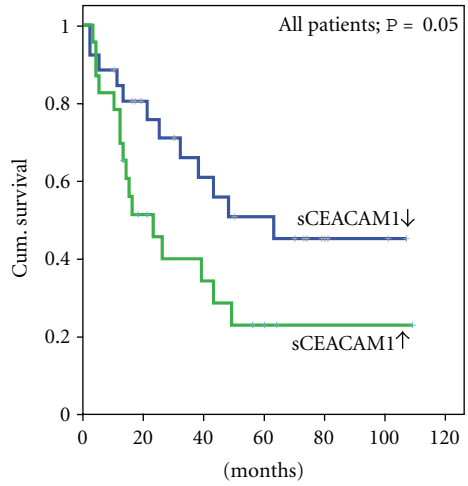
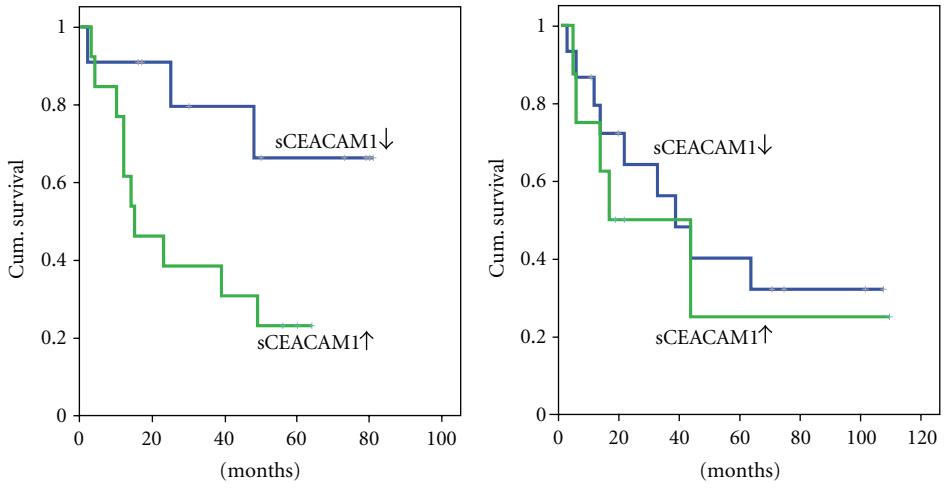


Figure 3: The change of sCEACAM1 following vaccination inversely correlates with survival. The change in sCEACAM1 following treatment (post minus pre-vaccination) was calculated and the patients ($N = 49$) were divided according to the trend of sCEACAM1 change. Kaplan-Meier plots were used to describe the survival rates of each subgroup of patients. Groups sizes were as follows: $N = 26$ (decreased sCEACAM1) and $N = 23$ (increased sCEACAM1).

3.4. The Correlation of ΔsCEACAM1 with Survival Is Stronger than That of DTH Test. DTH (delayed-type hypersensitivity) skin reactivity using unmodified autologous melanoma cells is used to predict the ability of patients to develop an immune response against his/her tumor and is attributed to the vaccination procedure [39, 40]. Survival rate of the patients was analyzed in DTH-negative and DTH-positive groups, according to the trend in ΔsCEACAM1. Surprisingly, the DTH-negative group, that is, patients that were not expected to gain a survival benefit from the vaccine ($N = 24$), could be categorized according to ΔsCEACAM1 into two distinct prognostic groups (Figure 4(a)). Indeed, the 11 patients that exhibited a decrease in sCEACAM1 had a mean overall survival rate of 63 months, as compared to only 29 months in the 13 patients in which sCEACAM1 levels were increased (Figure 4, $P = 0.03$). In contrast, no significant differences were found between ΔsCEACAM1 subgroups in DTH-positive patients ($P = 0.58$, Figure 4(b)). These results indicate that CEACAM1 monitoring with ΔsCEACAM1 has an added and complimentary value to the DTH response test.

3.5. The Correlation Between sCEACAM1 and S100B. We have previously demonstrated that sCEACAM1 significantly correlates with LDH serum levels in metastatic melanoma patients [37]. Here, we analyzed the correlation between sCEACAM1 and another known melanoma serum biomarker, S100B. We could not observe a correlation between the absolute values of these two factors (data not shown). However, when categorizing values into high/low subgroups, we found a significant ($P = 0.02$) correlation between post-vaccination S100B and sCEACAM1 (Table 2), that is, low sCEACAM1 was most likely to be accompanied by



(a) DTH$^-$ patients; $P = 0.03$ (b) DTH$^+$ patients; NS

Figure 4: Monitoring of sCEACAM1 further stratifies DTH-negative patients into two prognostically distinct groups. The analysis described in Figure 3 was performed exclusively for (a) DTH-negative patients ($N = 24$) and (b) DTH-positive patients ($N = 23$). Subgroups sizes were $N = 11$ (DTH$^-$) or $N = 15$ (DTH$^+$) for decreased sCEACAM1 and $N = 13$ (DTH$^-$) or $N = 8$ (DTH$^+$) for increased sCEACAM1.

low S100B in the same patient, and vice versa. To conclude, sCEACAM1 correlated with disease state and with S100B and its dynamics over time highly correlated with overall survival rates.

4. Discussion

In this retrospective study, we examined 49 melanoma patients from advanced AJCC stages, before and following autologous vaccination, for serum sCEACAM1 levels. We found that sCEACAM1 increases over time in WED patients and that their sCEACAM1 levels are higher as compared to NED patients (Figure 1). This confirms our previously published results with a different cohort of melanoma patients and treatments [37]. As most NED and WED patients are at AJCC Stages III and IV, respectively, this result implies that sCEACAM1 could reflect disease burden. Indeed, sCEACAM1 is synthesized and secreted from cultured human melanoma cells and its concentrations correlate with the amount of seeded melanoma cells *in vitro* [37] as well as with tumor mass in mice (unpublished data). In addition, post-vaccination sCEACAM1 correlates with S100B (Table 2), which sensitively reflects tumor mass [9]. Collectively, this data further fortifies the possible value of sCEACAM1 in monitoring disease burden.

Importantly, high sCEACAM1 levels are likely to correlate with poor overall survival (Figure 2) and significantly discriminated between patients who died and patients who remained alive during follow-ups. However, to rule out the possibility that these observations stem entirely from the correlation of sCEACAM levels with disease burden (stage and evidence of disease) we analyzed the trend of sCEACAM1 change over time (post-vaccination minus pre-vaccination) for each of the patients. Remarkably, "increased sCEACAM1" patients had significantly poor overall survival rates as compared with "decreased sCEACAM1" patients, both in the whole group of patients (Figure 3) and in NED or WED patients subgroups. This indicates that monitoring of serum sCEACAM1 in melanoma patients has a prognostic predictive value. Furthermore, the majority of the patients in this cohort exhibited normal values of serum LDH (Table 1). This was not surprising, as the majority of the patients were either with no evidence of disease (29/49 patients) or with Stage III (10/49 patients). Blood marker levels were compared 6-7 m in average after initiation of treatment. None of the NED patients and most Stage III patients exhibited clinically evident progression during this period, which was supported by the normal LDH values. In contrast, sCEACAM1 levels enabled predictive stratification of the patients (Figures 2 and 3). It is therefore implied that in patients with normal LDH (mainly NED and Stage III), sCEACAM1 might have superior predictive value.

We have previously reported that autologous vaccination was associated with improved overall and disease-free survival in AJCC stage III melanoma patients who attained strong skin reactivity against their tumor cells [39, 40]. The decrease in sCEACAM1 in NED/Stage III patients following vaccination is in line with this data. Moreover, more "decreased sCEACAM1" patients were found among DTH-positive patients (15/23 = 65%) as compared with DTH-negative patients (11/24 = 46%), but this difference did not reach statistical significance, probably due to small population size. Interestingly, monitoring of ΔsCEACAM1 further identified two distinct prognostic subgroups ($P = 0.03$) among the DTH-negative patients, but not in the DTH-positive patients. It is implied that the change in sCEACAM1 during vaccination can identify more subtle, yet of prognostic importance, immune events that the crud skin test is unable to show. Therefore, sCEACAM1 has an added prognostic value to DTH test and both could be used in adjunct to achieve superior patient stratification.

Overall, the current data suggest that the alterations in serum sCEACAM1 levels in melanoma patients reflect disease activity and support its role as a reliable serum marker. This prognostic value could be derived from the reflection of disease burden by sCEACAM1, as described previously [37]. However, serum sCEACAM1 may be more than a biomarker and may also have a biological functional and play an active role in facilitating melanoma aggressiveness. The previous findings that sCEACAM1 is produced by active protein synthesis in melanoma cells and that its production does not result from protein cleavage [37] actually support this idea. Membrane-bound CEACAM1 protects melanoma cells from NK and T cells-mediated cytotoxicity and enable them to avoid immune attack. Expressed and secreted from melanoma cells but not from immune cells [37], sCEACAM1 might act in a similar manner as a soluble agonistic ligand, which activates membrane-bound CEACAM1 receptors on NK and T cells thereby inhibiting their effector functions. sCEACAM1 may also agonistically enhance other CEACAM1-mediated functions, such as angiogenesis. Alternatively, it antagonize membrane-bound CEACAM1 to inhibit the adhesive interactions between lymphocytes and activated endothelial cells, thus affecting the rolling, adhesion, and recruitment of lymphocytes. These hypotheses remain to be proven in future investigations.

The mechanism of sCEACAM1 production is currently unknown. It was shown in mice that removal of Exon 4 by alternative splicing generates a truncated protein due to a stop codon created at the junction between Exon 3 and Exon 5 [44]. A similar sequence analysis of the human *CEACAM1* shows that the junction between Exons 3 and 5 creates a new stop codon, thus sCEACAM1 may be formed as a result of specific alternative splicing. Revealing the cues that induce sCEACAM1 expression/secretion, as well as characterization of sCEACAM1 functional domains, will help in deciphering whether the intriguing sCEACAM1 protein harbors biological functionality.

Authors' Contribution

Dr. Markel and Dr. Lotem have contributed equally to this work.

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Review Article

More Than Just Tumor Destruction: Immunomodulation by Thermal Ablation of Cancer

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Over the past decades, thermoablative techniques for the therapy of localized tumors have gained importance in the treatment of patients not eligible for surgical resection. Anecdotal reports have described spontaneous distant tumor regression after thermal ablation, indicating a possible involvement of the immune system, hence an induction of antitumor immunity after thermoinduced therapy. In recent years, a growing body of evidence for modulation of both adaptive and innate immunity, as well as for the induction of danger signals through thermoablation, has emerged. Induced immune responses, however, are mostly weak and not sufficient for the complete eradication of established tumors or durable prevention of disease progression, and combination therapies with immunomodulating drugs are being evaluated with promising results. This article aims to summarize published findings on immune modulation through radiofrequency ablation, cryoablation, microwave ablation therapy, high-intensity focused ultrasound, and laser-induced thermotherapy.

1. Introduction

The local application of high or low temperatures is frequently used to induce protein denaturation, tissue necrosis, and tumor destruction in order to curatively or palliatively treat localized primary or secondary tumors [1]. Thermal ablative procedures in clinical practice comprise radiofrequency (RF) ablation, microwave ablation therapy (MWA), high-intensity focused ultrasound (HIFU), and laser-induced thermotherapy (LITT) with the use of high temperatures, as well as cryoablation with induction of low temperatures. Primarily all these techniques were applied for the palliative treatment of patients not eligible for surgical resection or frail patients with a reduced functional reserve capacity and many comorbidities [2, 3]. Local thermal ablative methods present several advantages as compared with surgery which include less damage to surrounding healthy tissue, greater patient comfort, for example, less pain and limitation in exercise due to wound healing, improved cosmetic results, and—in times of critical financial situations in the medical facilities—reduced cost and shorter periods of hospitalization [2, 4]. For selected patients, local thermoablative techniques have similar clinical outcomes as compared with historical controls of surgical resection [5–8]. However, except for early hepatocellular carcinoma, no large randomized clinical trial has been performed to directly compare thermoablation and surgical resection so far [9]. In clinical routine, thermal ablation techniques have gained further importance in the treatment of small tumors as an alternative to surgical resection. Their application is limited by the size of the tumor lesions since large tumors (>4 cm) require more expanded treatment with an increased rate of complications and local recurrence [10, 11].

The choice of the most suitable thermal ablation modality depends on different premises. Tumors located in tissues with a high impedance like lung or bone can be better

treated with cryoablation or MWA [12–14]. Other factors for the assignment to an ablation modality depend on patient characteristics and comorbidities, on the physician's choice and availability of a certain method in a respective hospital, as well as on tumor location and relative position to other anatomic structures [1]. The clinical indications and characteristic features of each technique are summarized in Table 1.

The concept of thermal treatment for cancer is not new. The first patients with cerebral tumors were already treated with RF ablation in the early 20th century, but it took until the 1990s for RF ablation to become an accepted, commonly used treatment option for primarily unresectable tumor lesions in liver, kidney, bones, and lung [15]. During RF treatment, one or more RF applicators are placed in the target tissue and high-frequency alternating current is generated, leading to frictional heating above 60°C up to 100°C inducing coagulative necrosis [2, 16]. Higher temperatures would result in desiccation and subsequent increase in tissue impedance which limits further conduction of electricity into the tissue [12]. Recent studies have shown that the clinical outcome after RF ablation is comparable or even better in comparison to that of surgical resection. Consequently, RF ablation is currently being discussed as a possible new standard for elimination of metastatic liver lesions and oligofocal hepatocellular carcinoma (HCC) [5, 8, 17] and further as a curative treatment option in HCC and metastatic stages of colorectal carcinoma (CRC) when combined with surgery [18, 19]. Early-stage non-small cell lung cancer (NSCLC) can also be successfully treated with RF ablation. However, retrospective comparative analyses of survival have shown a strong tendency to increased survival benefits for NSCLC patients treated with surgery compared to RF ablation (46 versus 33 months, $P = 0.054$) [20], limiting the application of RF ablation to patients with contraindications against surgery.

MWA represents a relatively new technique using electromagnetic waves to induce high temperatures of up to more than 100°C. Here also, an active microwave antenna is placed into the tumor. Since MWA does not require the conduct of electric current, temperatures above 100°C do not result in a decline of therapeutic efficacy [12]. This method could therefore be effectively applied in tissues with higher impedance like lung and bone [12]. In humans, MWA is currently mainly applied for the treatment of HCC [21, 22].

During HIFU, ultrasound beams of high energy are applied to focus acoustic energy on a well-defined region inducing tissue vibration. Although single ultrasound beams can penetrate tissue without causing significant heat, focussing beams from multiple directions into a selected region results in a temperature rise to over 60°C and subsequently in coagulative necrosis [23, 24]. HIFU also induces acoustic cavitation which represents an additional mechanical mechanism of tissue destruction. Acoustic cavitation (the expansion and contraction of gaseous nuclei in cells through acoustic pressure) leads to collapse of mitochondria, endoplasmic reticulum, as well as nuclear and cell membranes [25]. This procedure is the only noninvasive thermal technique and allows real-time imaging of the treatment progress by ultrasound (US) [25]. However, the clinical application of HIFU is limited since the size of the multidirectional ultrasound focus is confined by technical boundaries and a treatment time as short as possible is required for an accurate ablation [24]. HIFU has been applied for the treatment of breast, liver, pancreas, kidney, bone, prostate, and soft-tissue tumors [25–27].

Laser fibers placed into a tumor lesion are used for laser ablation where photon energy conduction induced heating can reach temperatures of over 50°C. The tissue penetration depth of laser light is only 0.4 mm which implies that multiple laser fibers have to be positioned into a tumor to ensure optimal tissue destruction [28]. However, this limited penetration can facilitate the monitoring and accuracy of the ablation. This technique is experimentally used for the treatment of breast, brain, liver, bone, and prostate tumors [29, 30]. More extensively used is the clinical exertion of laser photocoagulation in retinal diseases, here also leading to retinal scarring [31].

In contrast to all the techniques mentioned above, cryoablation utilizes not high, but extremely low temperatures that sink to −160°C [32]. Cryoablation involves the evaporation of liquid gases and is a purely thermal process which does not require application of electrical current, leading to a broader applicability in high impedance tissues like lung or bone. The extent of tissue destruction can be easily monitored by direct monitoring of *ice-ball* formation with all conventional imaging modalities [1]. Cryoablation is used in broad clinical application, even for the treatment of retinoblastoma in children [33]. In 1–6% of cases cryoablation causes a systemic inflammatory response syndrome (SIR), termed as the *cryoshock phenomenon* which represents a potentially life-threatening complication [34–38] and limits its clinical application, especially for liver tumors [39].

The observation that spontaneous regression of untreated tumors can occur after thermoablation of distant tumor masses may indicate an involvement of immune activation upon thermoablation [39–42]. The initiation, maintenance, and termination of an effective antitumor immune response requires a complex interplay between cellular (immune cells including effector and regulatory subsets) and humoral components (cytokines, chemokines, antibodies). Various constitutive or inducible danger signals released by injured cells are known to play a determinant role in alarming the immune system against self-damage. In this *danger model*, cells dying by physiological processes such as apoptosis will be rapidly eliminated and ignored by the immune system whereas necrotic cells releasing their content in the extracellular space will trigger an immune response [43, 44]. In particular, heat shock proteins (HSP) constitute a group of molecular chaperones which stimulate the maturation of dendritic cells (DC) and carry antigenic peptides from their cells of origin inducing subsequent priming of antigen-specific T cells [45–48]. Local ablative treatment induces necrosis which may naturally modulate all of these parameters by inducing inflammatory processes finally leading to the development of an antitumor specific immune response. A growing series of reports describing inflammatory responses, antigen release

Table 1: Thermal ablative methods in clinical use for the treatment of cancer and described effects on the immune system.

			Immune Modulation			
Treatment	Indication	Characteristics/principle	Component	Effect	Ref.	Species
Radiofrequency (RF) ablation	*Clinical indication:* primary and secondary malignancies in liver, kidney, lung, and bone [2, 8, 17, 49]	*Mechanism:* application of alternating RF current through tip applicator placed around and in tumor tissue resulting in heat and coagulative necrosis	Cytokines	+	[51–54]	Human
			Danger signals	+	[55–61]	Animal
				+	[62, 63]	Human
			Granulocytes	+	[64, 65]	Animal
					[54, 66]	Human
	Experimental application: tumors of the breast [50]		NK cells	+	[61]	Animal
		Approach: percutaneous, open, and intraoperative		+	[67]	Human
			Monocytes/ Macrophages	+	[66]	Animal
		Image guidance: US, CT, and MRI	DC	+	[68, 69]	Animal
					[51]	Human
			T cells*	+	[49, 57, 65, 68–74]	Animal
				+	[70, 75–78]	Human
			T_{reg}	−	[54]	Human
			B cells	+	[75]	Human
			Antibodies*	+	[78]	Human
Cryoablation	*Clinical indication:* primary and secondary malignancies in liver, kidney, and prostate, as well as dermatologic and ophthalmologic tumors [4, 33, 79, 80].	*Mechanism:* application of cold through gaseous evaporation at the tip of a cryoprobe. Repetitive freezing and thawing cycles lead to direct cellular damage through ice crystals, vascular and endothelial injury, and eventually thrombosis and ischemia [79, 82] resulting in coagulative necrosis and apoptosis at the ablation margin	Cytokines	+	[39, 83]	Animal
				+	[36, 84, 85]	Human
			Danger signals	?		
			Granulocytes	+	[86]	Animal
			NK cells	+	[83]	Animal
	Experimental Application: tumors of the breast [81].			+	[87]	Human
			Monocytes/ Macrophages	+	[86, 88]	Animal
			DC	+	[69]	Animal
			T cells	+	[38, 69, 79, 83, 89–98]	Animal
		Approach: percutaneous, open, intraoperative		+	[85, 87, 99–102]	Human
			T_{reg}	+	[103, 104]	Animal
				−	[105]	Human
		Image guidance: US, CT, and MRI	B cells	+	[91]	Animal
			Antibodies*	+	[86, 106–113]	Animal
				+	[41, 87, 114–116]	Human
Microwave ablation therapy (MWA)	*Clinical indication:* mainly used for treatment of HCC, but also other primary and secondary malignancies of the liver [21, 22]	*Mechanism:* application of microwaves through tip applicator leading to coagulative necrosis [21]	Cytokines	?	[117]	Animal
			Danger signals	+		
			Granulocytes	?		
			NK cells	+	[118]	Animal
		Approach: percutaneous, open, and intraoperative		+	[119]	Human
			Monocytes/ Macrophages	+	[119]	Human
			DC	?		
		Image guidance: US, CT, and MRI	T cells	+	[118]	Animal
				+	[22, 119, 120]	Human
			T_{reg}	?		
			B cells	+	[22]	Human
			Antibodies	?		

Table 1: Continued.

			Immune Modulation			
Treatment	Indication	Characteristics/principle	Component	Effect	Ref.	Species
High-intensity focused Ultrasound (HIFU)	*Experimental application:* primary and secondary malignancies in breast, liver, pancreas, kidney, bone, prostate, and soft-tissues-tumors [121]	*Mechanism:* application of focused ultrasound beams of high-intensity resulting in coagulative necrosis	Cytokines	?		
			Danger signals	+	[122]	Human
			Granulocytes	?		
			NK cells	+	[123]	Human
			Monocytes/ Macrophages	?		
		Approach: noninvasive				
			DC	?		
		Image guidance: noninvasive real-time US	T cells	+	[121, 124]	Animal
				+	[27, 125, 126]	Human
			T_{reg}	?		
			B cells	+	[27]	Human
			Antibodies	?		
Laser induced thermotherapy (LITT)	*Clinical indication:* broadly applied for photocoagulation in retinal disease [127], primary, and secondary malignancies of the liver [128]	*Mechanism:* placement of multiple simultaneous fired laser fibers into a tumor resulting in coagulative necrosis [129]	Cytokines	+	[130]	Human
			Danger signals	+	[131]	Animal
			Granulocytes			
			NK cells	?		
	Experimental application: primary and secondary malignancies of the breast, brain, bone, and prostate [129]	*Approach:* percutaneous	Monocytes/ Macrophages	?		
			DC	?		
		Image guidance: MRI, CT, and US	T cells	+	[128, 132]	Animal
			T_{reg}	?		
			B cells	?		
			Antibodies	?		

Asterisks indicate allocation of T-cell or antibody responses to defined antigens.
Ref., reference number.

and uptake by professional APC and antitumor adaptive immunity shows that this can indeed be the case. This review aims to summarize findings on the modulation of the immune system through high- or low- temperature-induced thermal tissue ablation of cancer in animal tumor models and cancer patients. The respective techniques are presented in the order of common clinical use.

2. Radiofrequency Ablation

RF ablation has the broadest application in cancer treatment. It is therefore not surprising that most recent data relating to the activation of the immune system through thermoablation have been obtained using this method (Table 2).

2.1. Cytokines and Stress Response. Several groups have evaluated the systemic release of cytokines, chemokines, and various stress factors after RF ablation. Serum levels of proinflammatory cytokines like interleukins IL-1β, -6, and -8, as well as TNF were found to be either increased [51–54] or unchanged [133, 134]. In general, changes were modest and transient (several hours to days after ablation) [38, 52, 53, 134]. Moreover, IL-10 could be elevated in the serum postinterventionally [54, 133]. Over all, no case of severe SIR with multiorgan failure and coagulopathy, but significant increases in body temperature, mean arterial blood pressure, and concomitantly increased serum levels of adrenaline, nor adrenaline or C-reactive protein (CRP) have been reported shortly after RF ablation [53, 54, 133, 134].

In murine models, RF ablation induced strong upregulation of mRNA and/or protein levels of HSP-70, HSP-90, and glycoprotein 96 (gp96) as well as translocation of nuclear high-mobility group protein B1 (HMGB1) into the cytoplasm of tumor cells and into the intercellular space [55–57]. More specifically, increased HSP-70 expression was shown to occur at the margin of the ablation zone, the so-called transition zone, both in animals [55, 58, 59] and in human liver cells *in vivo* [62]. The time frame of maximal HSP-70 expression is described to be no more than 24 hours after ablation [56, 60, 62], the protein remaining upregulated in the necrosis surrounding tissue three days after RF ablation [61]. Several factors may influence local expression of heat shock proteins after RF: in rats receiving thermal ablation in different zones of the liver, the degree of HSP-70 expression was observed to be dependent on the relative spatial position of the ablated area to larger liver vessels since the blood stream of these vessels can nourish adjacent cells preserving cellular metabolism and, hence, expression

Table 2: Studies reporting immune modulation in cancer patients and animal models treated with RF ablation.

Species	Tumor	Model	Immunologic effect	References
Human	HCC ($n = 1$)		HSP-70, HSP-90 (cytoplasm, membrane)↑	[62]
	HCC ($n = 8$)		Activation of myeloid dendritic cells (blood) IL-1β, TNF (serum)↑	[51]
	HCC ($n = 20$)		$CD4^+$ and $CD8^+$ cells (blood)↑	[70]
			$CD3^-CD56^+$, $CD56^+CD16^+$ cells (blood)↑	
			Activity of tumor-specific T cells↑	
	HCC ($n = 37$)		$CD3^-CD56^{dim}$ cells (blood)↑	[67]
			Activity of $CD3^-CD56^{dim}$ cells↑	
	HCC ($n = 20$)		Tumor-antigen specific T cells (blood)↑	[77]
	RCC ($n = 6$)		$CD3^+$HLA-DR^+, $CD4^+$ and $CD8^+$ cells (blood)↑	[71]
			$CD56^+CD16^+$cells (blood)↓	
	Liver metastases ($n = 8$) HCC ($n = 4$)		Neutrophils (blood)↑	[66]
	Liver metastases ($n = 6$) HCC ($n = 6$)		$CD4^+$ and $CD8^+$ cells (blood)↑	[76]
	Liver metastases of CRC ($n = 10$)		IL-6 (serum)↑	[52]
	Liver metastases ($n = 9$) HCC ($n = 2$)		IL-6 (serum)↑	[53]
	Liver metastases ($n = 13$) HCC ($n = 4$)		$CD4^+$ cells (blood)↓ MUC-1 specific T cells (blood)↑ B cells (blood)↑ (only in metastatic cancer patients) Trafficking of $CD62L^+$ T cells into tissues	[75]
	Lung metastases ($n = 4$) NSCLC ($n = 10$)		IL-8, MIP-1α, MIP-1β (serum)↑ IL-10 (serum)↑ $CD4^+CD25^+Foxp3^+$ cells (blood)↓ Neutrophils (blood)↑	[54]
	Liver metastases ($n = 13$) HCC ($n = 4$)		IL-6 (serum)↑ IL-10 (serum)↑	[133]
	Metastases ($n = 29$) Primary tumors ($n = 26$) Metastases ($n = 16$)	± chemotherapy	$CD4^+$ and $CD8^+$ responses against tumor-specific antigens (blood)↑ Tumor-specific antibodies (serum)↑	[78]
	HCC ($n = 4$) RCC ($n = 2$)		HSP-70 (serum)↑	[63]
Mouse (BALB/c)	CRC	CT26 hEpCam ± huKS-IL2	Antitumor activity (splenocytes)↑ Tumor growth (distant tumor)↓ Tumor growth (rechallenge)↓	[74]
		C26	$CD4^+$ cells (perinecrotic)↑ Neutrophils (perinecrotic)↑ Neutrophils and lymphocytes (distant metastases)↑	[65]
	HCC	BNL ± CCL3	$CD11c^+$ cells (blood)↑ $CD11c^+$ cells (tumor)↑ $CD4^+$ and $CD8^+$ cells (tumor)↑ Tumor-specific cells (tumor)↑ Tumor growth (distant tumor)↓ Tumor growth (rechallenge)↓	[73]

Table 2: Continued.

Species	Tumor	Model	Immunologic effect	References
Mouse (C57BL/6)	Melanoma	B16-OVA ± CTLA4-mAb ± T_{reg} depletion	$CD8^+$ tumor-antigen specific T cells (blood)↑ Tumor growth (rechallenge)↓	[72]
		B16-OVA ± CTLA4-mAb	Antigen loaded DC, DC maturation (draining lymphnodes)↑	[69]
			Tumor growth (rechallenge)↓	
		B16-OVA ± DC	HSP-70, gp96 (tumor)↑	[57]
			HMGB1 (tumor)↑	
			$CD8^+$ tumor-specific T cells (spleen, draining lymph nodes)↑	
			Local recurrence↓	
			Tumor growth (rechallenge)↓	
	Urothelial carcinoma	MB49 ± DC	$CD4^+$, $CD8^+$ antitumor responses (splenocytes)↑	[49]
			$CD11c^+$cells (tumor)↑	
			Tumor growth (rechallenge)↓	
Mouse (NIH (S)-nu)	CRC	HT29	HSP-70 mRNA (cytoplasm)↑	[56]
Rabbit	Hepatoma	VX2	Lymphocytes, plasma cells, and neutrophils (tumor)↑	[68]
			Tumor-specific T cells (blood)↑	
Rat (Fisher)	Mammary	MatBIII	$CD161^+$ cells (tumor-surrounding tissue)↑	[61]
			HSP-70 (tumor-surrounding tissue)↑	
		R3230 ± liposomal doxorubicin	HSP-70 (around central coagulation zone)↑	[59]
Rat (rNU)	Hepatoma	SK-HEP-1	HSP-70, HSP-90 (cytoplasm, membrane)↑	[55]

of HSP in these cells [60]. Further experiments in nude rats transplanted with human HCC also suggested a correlation between applied energy and level of expression of HSP-70 and -90 [55]. As we recently described, a significant systemic release of HSP-70 into the serum can also be detected one day after treatment in RF-treated cancer patients, but serum levels did not correlate with ablation volumes, histological tumor type, and other clinical or laboratory parameters [63].

2.2. Cellular Immunity

2.2.1. Changes in Peripheral and Intratumoral Immune Cell Subsets. Postinterventional changes in peripheral leukocyte subsets have been observed by several groups and taken as evidence for the immune modulatory effect of RF ablation. Of note, antibody tools for cell subset identification, timepoints of observation, and patient cohorts differed between published studies. A decrease of circulating $CD4^+CD25^+Foxp3^+$ regulatory T cells (T_{reg}) was observed in patients 1 month after RF ablation of lung nodules [54]. In another study including 20 HCC patients, no significant changes in T-cell subsets were detected 1 month after RF (naïve or memory $CD4^+$, $CD8^+$) while increased percentages of activated T cells and circulating NK cells were noted in randomly selected patients from the study cohort [70]. The same group later described a marked expansion of $CD3^-CD56^{dim}$ effector NK cells 1 week and 4 weeks after treatment [67]. Matuszewski and colleagues evaluated lymphocyte subpopulations after RF ablation of renal cell carcinoma (RCC) in 6 patients and found a globally increased proportion of activated T cells in the majority of patients ($CD3^+HLA\text{-}DR^+$) whereas effects on $CD4^+$, $CD8^+$, and NK ($CD56^+CD16^+$) cells varied among individuals and at different timepoints [71]. In patients with colorectal liver metastases, but not with HCC, a transient decrease in $CD3^+CD4^+$ T cells was noted shortly (day 2) after treatment [75]. Although these and further observations are heterogenous, they collectively suggest an impact of RF ablation on various peripheral cell subsets, including T and NK cells [61], but also neutrophils, monocytes, B lymphocytes, and even DC [51, 54, 66, 75].

The assessment of tumor-infiltrating cells before and following RF ablation is intrinsically difficult in patients and available data have been obtained in various animal models. Most reports describe infiltration of immune cells in the transition zone hours to days after treatment. Granulocytes, macrophages, plasma cells, DC, $CD3^+$, and $CD4^+$ cells were found [49, 64, 68]. Interestingly, neutrophils and lymphocytes could also infiltrate distant, untreated metastases [65].

2.2.2. Antitumor Specific Responses. Few data addressing the adaptive immune response to tumors after RF ablation are available.

In a transplant-tumor model of VX2-hepatoma, rabbits were randomly assigned to treatment with RF ablation or to observation. Two weeks after RF ablation, the activation

of tumor-lysate specific T cells was detected and persisted over a postinterventional observation period of 6 weeks [68]. Animals in the RF-treated group had a significant survival increase [68].

Antigen-specificity of RF-induced antitumor T-cell responses was investigated in several reports. Dromi and coworkers used a murine urothelial carcinoma expressing the male minor histocompatibility antigen HY which was inoculated to female mice. T-cell responses against MHC-class I and class II HY-derived epitopes were significantly increased in the group of mice having received tumor RF ablation as compared to control animals. This was accompanied by an enhanced control of tumor growth, including upon rechallenge [49].

In a mouse model of OVA-expressing melanoma, adoptive transfer of splenocytes from RF-treated to naïve mice led to a growth retardation of OVA^+-, but not OVA^--tumors after rechallenge, and to complete tumor elimination in 20% of the mice. The treatment could also induce long-lasting immunity since RF-treated mice surviving the first tumor inoculation were completely protected after a second challenge 70 days later [72]. Moreover, intratumor injection of tagged-OVA led to antigen uptake and maturation of $CD11c^+$ cells in the tumor-draining lymphnode, albeit to a lesser extent than after cryoablation which was directly compared to RF ablation in this model [69].

In patients, HCC-reactive T cells were detected with IFNγ ELISPOT in 4/20 patients before RF ablation upon stimulation of PBMC with lysate of autologous tumor cells obtained either before or after treatment. One month after RF treatment, cellular reactivity was observed in 9/20 patients, strongly suggesting an *in vivo* immunization effect after RF-intervention [70]. Similar results were reported in two further cohorts of HCC and CRC patients [76].

Three recent publications have addressed the antigen-specificity of the RF-induced T-cell responses in patients. Napoletano and colleagues detected an increased IFNγ production upon stimulation with MUC-1-derived glycopeptides in 2 patients treated for liver metastases and also an increase in circulating $CD3^-CD19^+$ B cells. However, the specificity of antibodies was not studied [75]. Hiroishi and coworkers investigated $CD8^+$ T-cell responses against MAGE-1, NY-ESO-1, and GPC3 antigens in patients with HCC and found that antigen-specific T cells were already detectable in samples obtained before RF ablation, and increased in approximately half of the patients [77]. Recently, we evaluated the occurrence of tumor-antigen specific T cells or antibodies after RF ablation in 55 cancer patients and found an increase in antigen-specific antibodies, and $CD4^+$ or $CD8^+$ T cells in several individuals receiving RF ablation alone or shortly after chemotherapy [78].

2.2.3. Combination Therapies. All the results presented above show that RF ablation is able to induce tumor-directed immunity; however, the observed therapeutic effects are limited. Combination therapies have therefore been already tested in preclinical models, with the aim to enhance antitumor responses and protection. For OVA-expressing melanoma, CTLA-4 blockade or T_{reg} depletion (with anti-CD25 mAb) showed improvement in tumor control and enhanced induction of OVA-specific $CD8^+$ T cells whereas CTLA-4 mAb application without RF ablation did not mediate the same effects [72].

The coadministration of the monocyte attracting chemokine ligand 3 and inflammatory protein-1α (CCL3/MIP-1α) [73], antibody-conjugated IL-2 [74] or even chemotherapy (liposomal doxorubicin) [59] also enhanced the effects of RF ablation. Finally, whereas intratumor injection of unloaded DC did not synergize with RF-treatment, application of tumor-lysate loaded DC was reported to abrogate tumor relapse in most animals. Interestingly, while vaccination with DC alone was ineffective with regard to survival benefits, the combination with RF ablation significantly improved the survival of tumor bearing mice [49, 57].

All these reports provide a strong rationale for testing the combination of RF therapy with immune-modulating agents in cancer patients. It has to be noted that—besides RF ablation—many patients currently receive additional therapies like chemotherapy which may also influence the development of tumor-specific immune responses as recently recognized [137].

2.3. Immune Response and Clinical Course. The relationship between occurrence of antitumor immunity after RF ablation and clinical outcome still remains elusive. In HCC patients with induced tumor-specific T-cell reactivity after RF ablation, the local- and distant-site recurrence was similar [70]. In contrast, Hiroishi and colleagues observed a correlation between the frequency of tumor-antigen specific T cells and a favorable tumor-free survival in HCC patients [77]. Here, it has to be noted that patients could additionally receive transarterial chemoembolization (TACE). We have recently observed a tendency to a better survival for patients who presented with at least a twofold increase of HSP-70 in the serum one day after treatment [63]. Since patient cohorts were small in all three studies, results need further confirmation.

3. Cryoablation

3.1. Special Premises of Cryoablation. While thermal techniques utilizing lethal high temperatures have been so far mostly described to stimulate immune responses, cryoablation has been described to exert both stimulatory and suppressive effects on the immune system. These particular features could be due to the specific physiological mechanisms of cold injury including (i) direct cellular damage through formation of ice crystals, and (ii) vascular and endothelial injury with potential ischemia [82]. Whereas most other thermoablative techniques are believed to induce essentially coagulative necrosis, apoptotic cells might be also present at the outer rim of the ablation zone after cryoablation. According to the *danger model*, apoptotic cells do not release their cellular content (antigens, HSP, and HMGB1) and induce immunological tolerance [43, 44].

It has been proposed that larger numbers of apoptotic cells might cause tissue protection and lead to immunosuppression while larger numbers of necrotic cells could serve as immunostimulators [4]. More recently, they showed that the cryoablation modality itself, that is, rate of freeze, influences both tumor growth and T cell recruitment [79]. Moreover, technique and rate of freezing cycles could play a role in the precise mechanisms of the watershed between immunosuppression and immunostimulation after cryoablation [79]. However, this model is questioned by more recent reports showing that apoptotic cells can also exhibit significant immunostimulatory capacity [138, 139]. One other influential factor for these contradictory observations could be the timepoint of immunomonitoring: early assessment might miss immune activation and antitumor activity. Interestingly, clinical improvement could be recorded rather late after cryotreatment (up to 10 weeks) [140, 141] which is in line with the new concept that assessment of tumor response upon immunotherapy should be performed later than after conventional cytostatic therapy [142].

Table 3 only presents recent immunological observations of the past decade. Many observations reporting immunosuppression by cryoablation were made earlier and are discussed below, but are not presented in the table.

3.2. Cytokines and Stress Response. Unlike the other thermoablative methods, cryoablation induces a cytokine release syndrome (SIR—1–6.4% of all cases, with a mortality rate of 0.2–4%) [34, 35], assimilated to the *cryoshock phenomenon* which is clinically manifested by thrombocytopenia, disseminated intravascular coagulation (DIC), and pulmonary failure [35–38]. Cryoshock is mainly limited to ablation of hepatocytes [39]. In sheep and rats, the frequency of SIR correlated positively with the extent of cryoablated liver tissue, animals with more than 35% of ablated tissue presenting an elevated risk of SIR [37]. Moreover, cryoablation leads to significant increases of serum IFNγ TNF, IL-6, and IL-12, but not IL-10 within several hours after intervention [36, 83, 84]. In a rat model, cytokine release after cryoablation, RF ablation, and LITT was compared. Between 1 and 6 hours after cryoablation, significantly elevated serum levels of IL-6 were observed. IL-10 serum levels were slightly, but not significantly elevated [38]. In patients, TNF and IFNγ could remain elevated for up to four weeks [85]. In a model of transgenic mice overexpressing HSP-70 only a slight increase of HSP-70 expression could be observed which proved to be tissue-protective against cryonecrosis in skeletal muscle cells. Here, is has to be noted that no complete cryoablation, but only cryolesioning of skin and skeletal muscle was performed [88]. To our knowledge, no data on HSP expression after necrosis induction through cryoablation are available.

3.3. Antibodies. The earliest reports on immune modification after cryoablation described autoantibody production against ablated normal and tumor tissues in rabbits and monkeys, as well as in patients [41, 106–112, 114]. These antibodies were essentially IgG and IgM in the serum [41, 86, 114] and at the vicinity of the ablated lesion mainly IgG and IgA [115] appearing within two weeks after intervention [41, 86, 114]. In contrast, Müller and colleagues treated osteosarcoma in mice with cryoablation and found a decrease of tumor-binding antibodies [113].

Another effect of cryoablation was detected by Ravindranath and colleagues who observed a release of gangliosides into the circulation of CRC patients after cryoablation but not after RF ablation or surgery. At the same time, the group also described increasing titers of anti-ganglioside IgM antibodies [116]. Since anti-ganglioside antibodies have inhibitory effects on primary tumors, such as the induction of complement mediated killing [143] or apoptosis [144], production of antitumor antibodies might be one of the mechanisms underlying the immune-mediated tumor rejection following cryoablation [116].

3.4. Cellular Immunity

3.4.1. Changes in Peripheral and Intratumoral Immune Cell Subsets. In rats, significantly elevated peripheral leukocyte counts—especially $CD3^+$ and $CD4^+$ T cells—were detectable between 1 and 14 days after intervention [38, 89]. In humans, cryoablation led to an increase of circulating T cells in few patients [99, 100]. In a randomized trial, cryoablation—compared to conventional surgery—led to increased numbers of helper T cells and activated T cells [101]. In a cohort of patients with liver metastases, an increase of the Th1/Th2 ratio was observed in the peripheral blood [84] whereas Zhou and colleagues reported a decrease of circulating $CD4^+CD25^+Foxp3^+T_{reg}$ after cryolesioning of HCC [105].

In tumor draining lymph nodes (TDLN), increased cellularity was observed both in T-cell (paracortical) and B-cell (germinal center) areas one week after treatment. Immunologic activity could remain increased over a time span of up to 10 weeks [90, 91]. Using a xenograft model of human melanoma in nude mice, Gazzaniga and coworkers further described a massive intravascular and peritumoral recruitment of leukocytes, essentially neutrophils and macrophages after cryoablation [86]. In a mouse mammary cancer model, the number of $CD4^+$ T cells in TDLN was augmented. Interestingly, $CD4^+CD25^+T_{reg}$ were more numerous after low rate freeze [79].

3.4.2. Antitumor Specific Responses. Assessment of the immune modulation by cryoablation has yielded contradictory results. Older works have pointed out immunosuppressive effects: an increase of circulating immune effector cells was not of functional relevance for tumor rejection, rather, tumor outgrowth and increased metastasis was promoted. This indicated that cryoablation might mediate deleterious effects possibly by induction of suppressor T cells, today referred to as regulatory T cells, as well as delayed development of antitumor immunity [103, 104, 145]. In line with these findings, Machlenkin and colleagues did not observe cellular activation through cryotherapy as a monotherapy [92].

Table 3: Recent Studies reporting immune modulation in cancer patients and animal models treated with cryoablation.

Species	Tumor	Model	Immunologic effect	References
Human	CRC ($n = 110$)		Gangliosides (GM_2, GD_{1a}, GT_{1b}; serum)↑ Antiganglioside antibodies (serum)↑	[116]
	HCC ($n = 111$)		$CD4^+CD25^+Foxp3^+$ cells (blood, ablation zone surrounding tissue)↓	[105]
	Prostate ($n = 20$)		IFNγ ↑, TNF (serum)↑ Tumor-specific T-cell responses (blood)↑	[85]
	Prostate ($n = 12$)	± GM-CSF	Tumor-specific T-cell responses (blood)↑	[85]
	RCC ($n = 6$)	+ GM-CSF	Tumor-specific T-cell responses (blood)↑ Tumor-specific antibodies (serum)↑	[87]
	Liver metastases ($n = 12$) CCC ($n = 3$)		IL-6, TNF (serum)↑ Th1/Th2 ratio (blood)↑	[84]
Mouse (BALB/c)	CRC	Colon-26 ± krestin	$CD8^+$ antitumor T-cell reactivity (spleen) (↑) Number of metastases↓	[94]
		Colon-26 ± T_{reg} depletion ± DC + BCG	Tumor-specific $CD8^+$ T cells (spleen)↑ Tumor growth (distant tumors)↓	[96]
		Colon-26 ± cyclophosphamide	Tumor-specific T cells (spleen, draining lymph nodes)↑ Tumor growth (rechallenge)↓	[97]
	Mammary	MT-901	IFNγ, IL-12 (serum)↑ Tumor-specific T cells (draining lymph nodes but not in spleen)↑ NK cell activity (spleen)↑ Tumor growth (rechallenge)↓	[83]
			T cells (draining lymph nodes)↑ Tumor-specific T cells (draining lymph nodes)↑ Pulmonary metastases↓	[95]
			Tumor-specific T cells (draining lymph nodes)↑ Pulmonary metastases (high-intensity freezing)↓ Pulmonary metastases (low-intensity freezing)↑	[79]
	Melanoma	B16-OVA ± imiquimod	Tumor-specific T cell proliferation↑ Tumor growth (rechallenge)↓	[98]
		B16-MO5 ± DC	Tumor growth (rechallenge)↓	[92]
Mouse (C57BL/6)	Melanoma	B16-OVA ± CTLA4-mAb	DC maturation and antigen uptake (TDLN)↑ Tumor growth (rechallenge)↓	[69]
		B16-OVA ± CpG	DC (TDLN)↑ $CD4^+$, $CD8^+$ T cells (TDLN)↑ OVA-specific T cells (TDLN)↑ Tumor growth (rechallenge after peritumoral CpG administration)↓	[135]
Mouse (NIH (S)-nu)	Melanoma	IIB-MEL-J (human) ± GM-CSF	Neutrophils ($RB6\text{-}5CG^+$) (tumor-surrounding tissue)↑ macrophages ($F4/80^+$; tumor-surrounding tissue)↑ DC ($DEC205^+$; tumor-surrounding tissue)↑	[86]

Table 3: Continued.

Species	Tumor	Model	Immunologic effect	References
Mouse (OT-I T cell receptor (Vα2/Vβ5) transgenic)	Lung	Lewis lung tumor D122 ± DC	Tumor-specific $CD8^+$ T-cell proliferation↑ Th1 responses↑ Tumor growth (lung metastases)↓	[92]
Rat (Wistar)	Glioma	C6	$CD3^+$ and $CD4^+$ T-cell percentages (blood)↑ $CD4^+/CD8^+$ ratio (blood)↑	[89]

(↑) Weak induction.

In contrast, other groups demonstrated immunologic activation in cryotreated animals (Table 3). Kimura and colleagues found an increased cytotoxic activity of peripheral lymphocytes and splenocytes against a murine leukemia virus-induced lymphoma [146]. Regression of distant metastases and resistance to tumor rechallenge was described by Bagley and colleagues who found that splenic lymphocytes isolated from sarcoma-bearing mice treated with cryoablation exhibited significantly increased cytotoxic activity against sarcoma cells as compared to those obtained from mice undergoing limb amputation [93]. Increased immunological activity could be delayed up to ten weeks after intervention [140].

Urano and colleagues observed an increased activity of tumor-specific cytotoxic T lymphocytes (CTL) seven days after cryoablation in a mouse colon-carcinoma model. These effects were only observed after ablation of a single nodule while ablation of several lesions abrogated immune-related tumor regression. Here, a threshold of ablated tissue volume that governed immune stimulation or suppression was proposed [94]. Interestingly, tumor-specific effector cells isolated from TDLN but not from the spleen or peripheral blood secreted a higher amount of IFNγ (between days 3 and 7 after treatment) as compared to cells obtained following surgical resection.

In an OVA-expressing melanoma model, den Brok and colleagues observed an increase of antigen-loaded DC in draining lymph nodes both after cryoablation and RF-ablation. Of note, almost double as high cell numbers were observed compared to the induction through treatment with RF ablation [69]. Moreover, the numbers of infiltrating lymphocytes in TDLN were increased. These lymphocytes produced approximately 10-fold greater amounts of IFNγ upon stimulation with irradiated mammary adenocarcinoma cells after cryoablation than after surgical resection, delayed tumor growth and reduced the number of pulmonary metastases after adoptive transfer of TDLN cells of the cryoablated tumor [95]. The protection against a tumor rechallenge with B16-OVA cells was enhanced after cryoablation (50% surviving mice after 70 days) compared with the protective effect observed after RF ablation (20% surviving after 70 days) [79, 83, 95].

3.4.3. Combination Therapies. While Machlenkin and colleagues observed no clinical benefit with cryoablation alone, the combination with an intratumoral injection of immature DC induced robust activation of $CD4^+$ and $CD8^+$ CTL [92]. This synergistic effect was further improved after pretreatment with anti-CD4 or anti-CD25 mAb for T_{reg} depletion [96].

In an OVA-expressing melanoma model, CTLA-4 blockade and depletion of regulatory T cells could further enhance cryoinduced tumor-specific T-cell responses [69]. Alternatively, concomitant injection of CpG 1668, a TLR 9 ligand, had a similar effect on T-cell recruitment. The route of adjuvant injection was crucial for immune induction, peritumoral CpG application showing to be superior to distant site. Although tumor growth was delayed after combination therapy, survival benefit was not superior to treatment with cryoablation alone [135].

Conditioning with cyclophosphamide injected one day before cryoablation led to increased IFNγ production of tumor-antigen specific $CD4^+$ T cells in a mouse colon cancer model as detected in intracellular cytokine staining, enhanced survival and even some complete remission. Three out of four animals cured with the combination therapy also survived a tumor rechallenge with no macroscopically visible tumor upon autopsy [97]. Moreover, adoptive transfer of spleen and lymph node cells from surviving mice led to an improved survival in tumor-bearing mice. Depletion experiments showed that $CD8^+$ effectors were responsible for tumor elimination indicating that immunological memory had developed [97].

In the same OVA-expressing melanoma model used by den Brok and colleagues, Redondo and coworkers observed a clear survival advantage for mice treated with cryoablation combined with repeated topical application of imiquimod as an adjuvant indicating that TLR-7 activation can enhance tumor-specific immune responses induced by thermal treatment [98].

To sum up, all these reports suggest that combination of cryoablation with check point blockade or immunoadjuvants is a promising approach in the treatment of cancer patients.

3.5. Immune Response and Clinical Course. In patients with hormone refractory prostate carcinoma, a combination of cryoablation with injection of GM-CSF as adjuvant was evaluated. T-cell reactivity against autologous tumor tissue lysates as determined in IFNγ ELISPOT was found to be weakly increased after therapy, and no correlation could be established between the breadth of the immune response and the clinical course as measured by analysis of PSA serum levels [85, 102]. Also in a small cohort of patients with RCC,

increased cytotoxic T-cell activity and increased antitumor serum antibodies in selected patients were observed which only weakly correlated with a favorable clinical response. Here also, GM-CSF was applied as an adjuvant [87].

4. Microwave Ablation

As with RF ablation, microwave ablation therapy (MWA) induces hyperthermia leading to coagulative necrosis. The clinical application of MWA is, however, more limited than the thermoablative methods discussed above and only few groups have evaluated the immunomodulatory effects of MWA (Table 4).

4.1. Cytokines and Stress Response. MWA was described to induce HSP-70 expression in normal kidney tissue lysates obtained from treated rats, as detected with specific ELISA. However, HSP-70 expression was significantly lower upon MWA as compared to animals treated with RF ablation and cryoablation [117].

4.2. Cellular Immunity. In a mouse tumor model of HCC, only 2/10 animals experienced tumor rejection upon rechallenge after MWA, suggesting an existing but suboptimal protective antitumor immunity [118]. However, the protective effect could be improved by intratumoral coadministration of GM-CSF loaded microspheres, and even more by intraperitoneal CTLA-4 blockade. The triple combination not only led to rejection of newly inoculated tumors, but was also effective in the rejection of established distant tumors. Splenocytes isolated from the treated mice killed hepatoma cells *in vitro*, but not an unrelated tumor cell line. *In vitro* depletion experiments using mAb could further show that cytotoxicity was mediated by T cells (both $CD4^+$ and $CD8^+$) and NK cells, confirming that antitumor immunity was induced upon combination therapy [118].

One month after MWA, 10 patients with hypersplenism that had developed as a result of portal hypertension exhibited a transient peripheral increase of T helper cells ($CD3^+CD4^+$) and B cells, but not of cytotoxic ($CD3^+CD8^+$) T cells [22]. In a larger cohort of patients suffering from HCC, immune cell infiltration was studied by immunohistochemistry analyses of biopsy tumor samples taken either before or at different timepoints (3–30 days) after MWA application. A markedly increased infiltration of lymphocytes (predominantly $CD3^+$ T cells, $CD56^+$ NK cells, and macrophages, but not of B cells) was detected after MWA inside the ablated lesions, in the adjacent normal tissue and in distant untreated lesions [119].

4.3. Immune Response and Clinical Course. The density of infiltrates of lymphocytes, macrophages, and $CD56^+$ cells into MWA-treated liver tissue correlated inversely with the risk of local recurrence [119].

Zhou and colleagues performed a phase I clinical study in ten HCC patients with chronic hepatitis B by combining local microwave tumor ablation with immunotherapy, which was applied at 3 timepoints, that is, on the day of the MWA and then on days 11 and 100. Immature and mature monocyte-derived DC loaded with autologous tumor lysate were injected into the rim between the ablation zone and normal liver parenchyma and into the groin lymph nodes, respectively. Additionally, *in vitro* activated lymphocytes were applied intravenously. A modest and transient effect on peripheral T-cell subsets (decrease of $CD4^+CD25^{high}$—possibly T_{reg}—and increase of $CD8^+CD28^-$—differentiated $CD8^+$ T cells—was reported one month after treatment concomitant with a reduction in hepatitis B virus load observed in some patients, but analyses of the antitumor specific responses were not performed in this study. Of note, this clinical setting does not allow determining whether the observed effects were due to the MWA itself, to the immunotherapy regimen or to the combination of both treatments [120].

5. High-Intensity Focused Ultrasound (HIFU)

In addition to mere hyperthermia, HIFU also exerts nonthermal mechanistic constraints (acoustic cavitation) on treated tissues that might contribute to and modulate its effects on the immune system [122] (Table 5).

5.1. Cytokines and Stress Response. In breast cancer patients, increased HSP-70 expression was detected on the cell membrane of treated cancer cells. HSP expression was mainly found in the central necrosis zone while only a few positively stained cells were observed in the periphery [122].

5.2. Cellular Immunity

5.2.1. Changes in Peripheral and Intratumoral Immune Cell Subsets. In patients with posterior uveal melanoma [125], pancreatic carcinoma [123], osteosarcoma, HCC, and RCC [126] that were treated with HIFU, increased percentages of $CD4^+$ T cells and a higher $CD4^+/CD8^+$ ratio were observed [125, 126]. Another study observed only statistically significant higher NK cell percentages in the peripheral blood, while other leukocyte subsets remained stable [123].

In human breast cancer specimens collected 1-2 weeks after HIFU treatment, immunohistochemistry analyses showed a significant increase of T and B cells at the margin of the ablated region as compared to HIFU-untreated tumor samples. Interestingly, a subset of these cells were activated ($CD57^+$) and expressed perforin and granzyme B, indicating the presence of activated cytotoxic effectors [27].

5.2.2. Antitumor Specific Responses. In a model of experimental neuroblastoma, reduced secondary tumor growth after HIFU treatment was observed, although involvement of immune cells was not evaluated further [136].

Zhang and coworkers immunized mice with a vaccine consisting of a lysate of the H22 hepatoma cell line either untreated or pretreated *in vivo* with HIFU. Ten days after vaccination, animals received a subcutaneous tumor challenge. Tumor growth was significantly delayed in mice vaccinated with previously HIFU-treated tumor cells.

Table 4: Studies reporting immune modulation in cancer patients and animal models treated with MWA.

Species	Tumor	Model	Immunologic effect	References
Human	HCC ($n = 82$)		$CD3^+$ cells, $CD56^+$ cells (treated and distant tumors)↑	[119]
			$CD68^+$ cells (treated and distant tumors)↑	
	HCC ($n = 10$)	± DC	Phase I study:	[120]
			$CD4^+CD25^{high}$ cells (blood)↓	
			$CD8^+CD28^-$ cells (blood)↑	
Mouse (C57BL/6)	HCC	Hepa 1–6 ±GM-CSF ± CTLA4-mAb	Activity of tumor-specific $CD4^+$, $CD8^+$ cells (spleen)↑	[118]
			$NK1.1^+$ cells (spleen)↑	
			Tumor growth (rechallenge)↓	

Table 5: Studies reporting immune modulation in cancer patients and animal models treated with HIFU.

Species	Tumor	Model	Immunologic effect	References
Human	Breast carcinoma ($n = 23$)		HSP-70 (membrane)↑	[122]
	Breast carcinoma ($n = 48$)		$CD3^+$, $CD4^+$, $CD8^+$ cells (tumor)↑	[27]
			$CD20^+$ cells (tumor)↑	
			$CD57^+$ cells (tumor)↑	
	Pancreatic carcinoma ($n = 15$)		NK cells (blood)↑*	[123]
	Uveal melanoma ($n = 5$)		$CD4^+$ cells (blood)↑	[125]
	Osteosarcoma ($n = 6$)		$CD4^+$ cells (blood)↑	[126]
	HCC ($n = 5$)			
	RCC ($n = 5$)			
Mouse (Ajax)	Neuroblastoma	C1300 ± adriamycin	Tumor growth (rechallenge)↓	[136]
Mouse (C57BL/6J)	HCC	H22 ± DC	Activation of $CD8^+$ cells (spleen)↑	[124]
			Tumor growth (rechallenge)↓	
		H22 ± tumor lysate vaccine	Cytolytic activity (spleen)↑	[121]
			Tumor growth (rechallenge)↓	

* NK cell phenotype was not specified.

However, survival was not different between the vaccination groups [121]. In another model, the same group utilized a DC vaccine loaded with cell debris from HIFU-treated or -untreated tumor cells. While tumor growth was again reduced, no survival advantage could be observed. However, increased activity of $CD8^+$ splenocytes could be detected in IFNγ ELISPOT [124]. These were supported by *in vitro* experiments showing activation of bone-marrow derived DC upon incubation with tumor lysates and increased tumor killing by splenocytes harvested from HIFU-treated animals [121].

In contrast, several studies describe increased numbers of peripheral T cells following HIFU, which did not exert enhanced antitumor immunity [123, 125, 126].

Several groups observed a loss in tumor antigen expression in ablated prostate [23] or breast carcinoma [147, 148] lesions after HIFU. Such downregulation would be expected to lead to a reduced recognition of tumor tissue through antigen-specific T cells. Further investigations will be needed to determine whether the HIFU method is generally appropriate for efficient induction of antitumor T-cell immunity in patients.

6. Laser Ablation

Laser-induced thermotherapy (LITT) is applied widely for photocoagulation in retinal diseases, where the release of proinflammatory cytokines [127] or the activation of retina-specific T cells after panretinal photocoagulation (PRP) [149] have been described. In cancer patients, however, laser ablation is still experimental, and only scarce publications have addressed the modulation of cellular immunity through LITT (Table 6).

6.1. Cytokines and Stress Response. In patients, laser ablation led to increased levels of IL-6 and TNF-receptor 1 in the serum of patients suffering from primary and secondary malignant lesions of the liver 72 hours after treatment. Changes in the level of other proinflammatory cytokines such as TNF and IL-1β were not observed in this study [130].

In a murine model of colorectal liver metastases, LITT was also shown to enhance expression of HSP-70 at the margin of the coagulated tissue, with cytoplasmic and nuclear expression in sublethally damaged mouse hepatocytes and extraparenchymal cells. In tumor cells, this upregulation was

Table 6: Studies reporting immune modulation in cancer patients and animal models treated with LITT.

Species	Tumor	Model	Immunologic Effect	References
Human	CRC ($n = 4$) HCC ($n = 3$) Other ($n = 6$)		IL-6, TNF-R1 (serum)↑	[130]
Mouse (CBA)	Liver metastases of CRC	MoCR	HSP-70 (cytoplasm, nuclear)↑	[131]
	Subcutaneous CRC tumors	MoCR	$CD3^+$ cells (tumor-host interface)↑ Spontaneous IFNγ production (spleen, lymph nodes, tumor, and distant tumors)↑	[132]
Rat (WAG)	CRC	CC531	CD8, CD86, MHC-II, CD11a, and ICAM1 expression (invasion front of distant tumors)↑	[128]

detected between 12 hours and 7 days after intervention, with a peak at 24 hours [131].

6.2. Cellular Immunity. In WAG rats, Isbert and colleagues induced two independent tumors in the left and right liver lobes. One of the two tumors was either ablated with LITT or surgically removed, and immune cell infiltration into the untreated remaining tumor was compared to that observed in an untreated control group. Expression of CD8, CD86, MHC-class II, and adhesion molecules was found to be increased between 1 and 10 days after LITT at the tumor invasion front as compared to resection or no treatment, indicating an influx of immune cells [128]. Moreover, the growth of the untreated tumors was found to be considerably reduced in LITT-treated animals.

Using a murine CRC model, Lin and coworkers observed an increased infiltration of $CD3^+$ T cells into the tumor-host interface and into the tumor, as well as into the liver parenchyma and—to a certain extent—also into distant tumor lesions. Moreover, increased activation of splenocytes and tumor infiltrating lymphocytes was reported in *ex vivo* IFNγ ELISPOT without antigen restimulation [132]. Of note, these results were all obtained in animal models, and immune modulation after LITT has not been reported for cancer patients yet.

Taken altogether, the available results strongly suggest that laser therapy, as shown for other thermoablation methods, can stimulate antitumor immune effector cells *in vivo* [128, 132].

7. Conclusion and Perspectives: Implications for Anticancer Therapy

During the past two decades, numerous publications in animal models and patients have shown that local thermoablative techniques can induce or enhance tumor-specific immune responses that contribute to tumor control. Although the sequential mechanisms involved are not yet fully elucidated, several pieces of the puzzle have been identified: thermal treatment induces necrosis and can (i) lead to local inflammation, release of danger signals—for example, heat shock proteins—which may even been detected systemically; (ii) stimulate the recruitment and activation of immune effector cells, including DC, at the vicinity and most probably inside the damaged tumoral tissue. Both processes occur rapidly, that is, within a few hours to days following intervention; (iii) activate antitumor adaptive immunity, including $CD4^+$, $CD8^+$ T cells, and antibody production which can contribute to local tumor elimination, control distant tumors including micrometastases, and establish long lasting antitumor immunological memory [69, 72, 83, 93, 97, 121]. The source of tumor-associated antigens for inducing specific T cells may be either necrotic dying cells [138, 150, 151] or sublethally damaged cells [62, 63]. Besides these direct mechanisms, the removal of tumor tissue leads to depletion of T_{reg} and more generally may overcome local immunosuppression shifting favorably the balance towards effective antitumor immunity [61, 96, 105].

Hence, thermoablation can trigger physiological cascades necessary and sufficient for a protective immune response. Obviously, several methods can be applied successfully, suggesting that the key element is the induction of local necrosis, which can be achieved by using different settings and temperatures. High temperatures (RF ablation, MWA, HIFU, and LITT) seem rather to sustain antitumor activity whereas both immunomodulatory and immunosuppressive effects have been reported upon cryoablation. Of note, the lesions induced by high-temperature thermoablation are probably not solely of necrotic nature but may also exhibit apoptotic cells [16, 152, 153]. Whether opposite immunological outcomes are hence related to a different balance between apoptosis, necrosis, and secondary necrosis, with apoptotic cells acting more in a tolerizing or immunosuppressive fashion and necrotic cells more immunogenic, is at the moment unclear [138, 139]. Because necrosis induction can be easily visualized during thermoablative intervention, controlled necrosis might be an ideal tool for inducing enhanced immunogenic cell death [154]. Interestingly, local hyperthermia between 40°C and 44°C has been also been described to modulate immunity, as reviewed elsewhere [155].

However, it should be noted that the reported effects of thermoablation alone on the immune system are generally modest, suggesting that such treatment as a monotherapy is in general not capable of inducing sufficient immune responses for full tumor protection [97, 118]. Thermotherapy should be therefore most effective in case of a limited

tumor burden, ideally without detectable tumor postinterventionally, and not in advanced cancer where it is applied in most cases so far. Notably, combined therapies in order to enhance tumor-specific immune responses showed extremely promising results. Several strategies, such as check point blockade (anti-CTLA-4 mAb, T_{reg} depletion) [69] or application of adjuvants (interleukins or chemokines, GM-CSF, TLR agonists) have been evaluated in preclinical models but very little in clinical application yet. Randomized trials have still to be conducted.

In summary, thermal ablation represents a promising component for cancer immunotherapy in the treatment of small or subclinical tumor lesions which can be attacked by the patient's immune system. By controlled induction of physiological stress, it offers the possibility of letting the "natural" immune response develop in its whole by breaking self-tolerance. So, thermal ablation of cancer provides a therapeutic implementation of the *danger model*. However, the induced antitumor immunity is weak and probably not sufficient alone to eradicate established tumors, but it can synergize with some chemotherapies and immunomodulating strategies. Selecting the appropriate thermoablative method and finding optimal combinations for individual patients will be an exciting challenge for the upcoming years.

Abbreviations

CD	Cluster of differentiation
CRC	Colorectal carcinoma
CT	Computed tomography
CTLA	Cytotoxic T-lymphocyte antigen
DC	Dendritic cell
HCC	Hepatocellular carcinoma
HIFU	High-intensity focused ultrasound
HMGB1	High-mobility group protein B1
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
LITT	Laser-induced thermo therapy
mAb	Monoclonal antibody
MIP	Macrophage inflammatory protein
MRI	Magnetic resonance imaging
MWA	Microwave ablation therapy
n.a.	Not applicable
NSCLC	Nonsmall cell lung cancer
RCC	Renal Cell Carcinoma
RF	Radiofrequency
TDLN	Tumor draining lymph node
TLR	Toll-like receptor
TNF	Tumor necrosis factor
US	Ultrasound.

Conflict of Interests

None of the authors has any commercial interests or conflicts of interests to declare.

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Review Article

Monoclonal Antibodies in Gynecological Cancer: A Critical Point of View

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During the last decades, several improvements in treating gynecological malignancies have been achieved. In particular, target therapies, mostly monoclonal antibodies, have emerged as an attractive option for the treatment of these malignancies. In fact, various molecular-targeted agents have been developed for a variety of malignancies with the objective to interfere with a precise tumor associated receptor, essential for cancer cell survival or proliferation, blocking its function, of the cancer cells. Alternatively, monoclonal antibodies have been developed to block immune suppression or enhance functions of immune effector cells. So far, several monoclonal antibodies have been tested for clinical efficacy for the treatment of gynecological cancers. Antibodies against Vascular Endothelial Growth Factor (VEGF) and Epidermal Growth Factor Receptor (EGFR) have been used in different neoplasms such as ovarian and cervical cancer. Catumazumab, a bivalent antibody against CD3 and EpCAM, is effective in the treatment of neoplastic ascites. Other antibodies are peculiar for specific cancer-associated antigen such as Oregovomab against CA125 or Farletuzumab against the folate receptor. Here we describe the preclinical and clinical experience gained up to now with monoclonal antibodies in tumors of the female genital tract and trace future therapeutic and research venues.

1. Introduction

Despite the improvement achieved during the last decades in gynecological cancer treatment, most of these patients, especially women affected by ovarian cancer, are at great risk of recurrence and emerging drug resistance. Therefore, novel approaches are required to improve outcomes for gynecological cancer patients. Recently, various molecular-targeted agents have been developed and used in the management of a variety of malignancies, including ovarian, cervical, and endometrial cancers. The therapeutic benefits of targeted clinical interventions, with increased selectivity and fewer adverse effects, hold great promises in the treatment of solid malignancies, both as single therapy and in combination. In particular, Monoclonal Antibodies (MoAbs) represent the majority of target therapies which have been investigated and employed in clinical settings so far. These immunological reagents recognize molecular targets whose expression is tumor associated or/and are essential for the cancer cell survival and proliferation such as the Vascular Endothelial Growth Factor (VEGF), the Epidermal Growth Factor Receptor (EGFR) family, CA125, MUC1, and other signaling pathways which are aberrant in tumor tissue (EpCAM). Also, the targeting of immune cells by MoAbs has been proved to be an efficacious strategy to modulate immune system functions (anti-CTLA-4, anti-CD3, anti-CD40). To date, several MoAbs have been approved for the treatment of colorectal, breast, head and neck, nonsmall cell lung, and renal cell cancer (Table 1). Encouraging results have being achieved also in gynecological tumors. Here, we review the most promising MoAbs that are under early or advanced investigation for the treatment of neoplasms of the lower genital tract.

2. Rationale of Monoclonal Antibodies in Cancer Treatment

Significant advances in gynecological cancer management have been recently achieved, including interesting progresses in surgical, chemotherapeutic, and concurrent chemo-radioterapeutic settings. However, more effective, specific, and less toxic approaches need to be investigated. Based on the promising results of preclinical studies, various targeted therapies are currently being evaluated in cancer patients. One of the most promising approaches, that may improve patient outcome, is the use of MoAbs. The use of MoAbs in cancer treatment is focused on the idea of selectively targeting tumor cells that express tumor-associated antigen [1], with the aim to specifically antagonize receptor signaling pathways, which are essential for proliferation, survival, and migration of tumor cells. Thus, MoAbs offer increasingly customized solutions based on the targeting of multiple specific pathways essential for cancer development and metastasis by attacking targeted tumor cells. Furthermore, the high specificity of the target reduces cytotoxic side effects on normal tissue, seen with traditional chemotherapeutic agents, and should permit the maintenance of a high quality of life. The first experience of MoAb administration in cancer patient was carried out in a patient affected by non-Hodgkin's lymphoma [2]. Since then, several MoAbs against cancer-associated antigens have been developed and MoAbs have rapidly become one of the biggest classes of new drugs approved for the treatment of cancer (Table 1). To date, several ongoing trials are investigating the role of MoAbs in ovarian, cervical, and endometrial cancer (Tables 2–5). In some cases, MoAbs have already demonstrated favorable clinical outcomes in phase I/II studies and are being investigated further in phase III trials. However, further investigations for most of these molecules are required to establish a convincing proof of safety and efficacy of them in gynecological tumors.

3. Monoclonal Antibodies: Mechanisms of Action

MoAbs are antibodies produced by hybridoma cells. In the sixties the conventional route to derive MoAbs was to immunize mice. It took 10 years to be translated to the patient with MoAb muromonab, a murine-derived antibody for acute organ rejection approved by FDA in 1986 [3]. Recently, recombinant engineering techniques permitted the construction of MoAbs with possible variation in size, valence, configuration, and effectors functions. This technology results in the development of fragment, chimeric, humanized, and fully humanized MoAbs.

MoAb therapy consists in targeting specific extracellular/cell-surface pathways in order to destroy malignant tumor cells and prevent tumor growth by blocking specific cell receptors.

Binding specificity and selective molecular targeting are the major advantages of this approach. The general mechanism mediated by MoAb administration is the specific recognition of an antigen selectively expressed by tumor cells and the generation of immune-complexed cells, that can activate distinct immune mechanisms mainly mediated by the Fc region of the MoAb. Increased uptake by antigen presenting cells, NK activation, and induction of ADCC are the effects described. Moreover, the engineering of the Fc domain permits to increase affinity towards specific FcRs, potentiating the action of specific innate immune cells. This is the case of MoAbs directed against antigens that are homogenously overexpressed by cancer cells such as MUC1 and CA125. The identification of molecules that have a key role in tumor progression and immune modulation has led to the generation of immune reagents that combine specificity to the ability to exert a biological function on the target cell. Three main approaches can be identified.

(1) MoAbs Recognizing Specific Tumor-Associated Receptors. Tumor cells display specific receptors that are rare or absent on the surfaces of healthy cells, and which are responsible for activating cellular signal transduction pathways that cause the unregulated growth and division of the tumor cell. Specific targeting of these receptors can block tumor-associated transduction pathways, reducing tumorigenicity and invasiveness. MoAbs, such as trastuzumab, act through this mechanism [4].

(2) MoAbs Targeting Tumor Promoting Molecules. During tumor transformation several tumor promoting molecules are produced by the cancer cells, suppressing and subverting the function of immune system. The administration of MoAbs targeting such molecules can interfere in the binding of the molecule to its receptor or/and increases the clearence of these soluble factors, thus reducing tumor cell growth. Bevacizumab, the MoAb against VEGF molecule, is a paradigmatic example. In fact, binding of bevacizumab to VEGF blocks VEGF binding to its receptor [5].

(3) MoAbs Targeting Immune E ector Cells. The targeting of immune cells can be achieved by the employment of MoAbs specific for surface receptors that can have suppressing or activating function. The CTLA-4 molecule expressed by effector cells exerts an inhibitory function and the functional blocking of this molecule is being investigated in clinical trials [6].

On the other hand, targeting of activating molecules, such as CD3, is a strategy to enhance the functions of immune effector cells. The strategy utilized is to generate a bispecific antibody able to recognize simultaneously a relevant tumor antigen and an immune-specific activating molecule. In this way, the antibody combines the specific recognition of the target cells to the selective activation of the immune effector cell, bringing the two cells physically close, thus making easier the immune recognition. Catumaxomab, a bispecific antibody recognizing EpCAM and the CD3 molecule, is a prototype of such reagents [6].

Table 1: FDA-approved MoAbs for cancer patients.

Monoclonal antibody	Target	Approved cancer patients	Mode	Year of introduction
Rituximab	CD20	Non-Hodgkin Lymphoma	Chimeric IgG1	1997
Trastuzumab	ErbB2	Breast	Humanized IgG1	1998
Gemtuzumab-ozogamicin	CD33	Acute myeloid leukemia	Humanized IgG4 + ozogamicin	2000
Alemtuzumab	CD52	Chronic lymphocytic leukemia	Humanized IgG1	2001
Ibritumomab tiuxetan	CD20	Non-Hodgkin's lymphoma	Murine IgG1+Yttrium90	2002
I-Tositumomab	CD20	Non-Hodgkin's lymphoma	Murine IgG2a+iodine-131	2003
Cetuximab	EGFR	Colorectal Head/Neck	Chimeric IgG1	2003
Bevacizumab	VEGF	Colorectal	Humanized IgG1	2004
Panitumumab	EGFR	Colorectal	Humanized IgG2	2006
Ofatumumab	CD20	Chronic Lymphocytic Leukemia	Human IgG1	2009
Ipilimumab	CTLA-4	Late stage melanoma	Human IgG1	2011

4. Monaclonal Antibodies in Ovarian Cancer

In contrast to hematological malignancies and certain solid tumors such as breast and colorectal cancer, MoAbs have not been completely proven to be clinically effective in the treatment of ovarian cancer, although encouraging results are being achieving. Currently, the mostly investigated targets in ovarian cancer are (VEGF) and (EGFR) family members (EGFR1, EGFR2/ErbB2).

Other tumor-associated antigens, such as the adhesion molecule EpCAM, the epithelial mucins CA125 and MUC1, and the Folate Receptor as well as molecules expressed by immune cells such as CD3 and CTLA-4 are under evaluation.

4.1. Vascular Endothelial Growth Factor-Targeted Therapy: Bevacizumab

4.1.1. Vascular Endothelial Growth Factor (VEGF). VEGF, also known as Vascular Permeability Factor, is a potent angiogenetic cytokine that induces mitosis and regulates the permeability of endothelial cells.

Overexpression of VEGF correlates with increased microvascular density, cancer recurrence, and decreased survival in several neoplasms, including most gynaecological tumors [7–13].

In women with ovarian cancer, high serum levels of VEGF are found to be an independent risk factor for ascites, advanced-stage disease, undifferentiated histology, number of metastasis, and decreased survival [9–11]. In ovarian carcinoma there is suggestive evidence showing that higher VEGF levels are associated with aggressive clinical behavior.

4.1.2. Bevacizumab. Bevacizumab represents the most investigated target therapy in ovarian neoplasia. It is a humanized monoclonal antibody directed against the VEGF ligand able to inhibit the formation of new blood vessels and to decrease the diameter, density, and permeability of blood vessels, resulting in a normalization of tumor vascularization [14, 15]. Moreover it has been shown that VEGF acts as immunosuppressive factor, contributing to the skewing of the antitumor immune response and the development of immunosuppressive microenvironment [16]. Randomized trials in solid tumors have shown that the addition of bevacizumab to standard chemotherapeutic regimens results in statistically significant improvements in progression-free survival (PFS) and, in some cases, in overall survival (OS) [17–19]. Currently, bevacizumab has not been approved for any malignancy of the female genital tract, although initial encouraging data have been achieved for these types of neoplasms, especially for ovarian cancer. Up to day, several investigators have explored bevacizumab as a single agent or in combination with chemotherapy in the management of ovarian cancer [20]. Both alone or in combination with traditional drugs, it has shown interesting levels of activity and provided clinically meaningful results in patients with recurrent ovarian disease [21–30]. Furthermore, it has also been used as a palliative treatment of symptomatic ascites [21, 23, 31–35]. In one of these kinds of experience [35], immunological analyses after intraperitoneal bevacizumab administration showed a concomitant increase in number and function of CD8+ T effector cells and a decrease of circulating regulatory T cells (Treg) cells, similarly to what observed in ovarian cancer patients undergoing to debulking surgery and radiotherapy [36]. These effects observed on the immune performance of the patient can be due to the pleiotropic function of VEGF that can directly acts as immunosuppressive molecule on the immune microenvironment [16]. The most considerable results concerning the role of bevacizumab on progressive disease in ovarian cancer patients can be derived from recent analysis of two completed randomized phase III trials: the GOG 218 [37] and ICON 7 [38]. These were performed using bevacizumab in newly diagnosed advanced stage ovarian cancer, in association with standard chemotherapy. The GOG 218 [37] is a three-arm placebo controlled trial: 1873 patients have been randomized to iv paclitaxel-carboplatin for 6 cycles with or without bevacizumab in the latter five followed by placebo or additional 48 weeks of maintenance bevacizumab (15 mg/kg every 3 weeks). Preliminary data, initially presented at the 2010 meeting for the American Society of Clinical Oncology (ASCO), showed a significant improvement in PFS in patients treated with concurrent and maintenance bevacizumab, 14.1 months versus 10.3 months in the placebo arm. Relative to arm 1 of the trial, the hazard ratio for first

progression in the maintenance arm of the trial was 0.717 (95% CI: 0.625–0.824, $P < 0.0001$). OS data are not yet mature.

The ICON7 trial [38] is a two-arm, non-placebo controlled trial comparing carboplatin-paclitaxel (6 cycles) versus carboplatin-paclitaxel-bevacizumab (7,5 mg/kg) every three weeks for 6 cycles, followed by 12 cycles of maintenance bevacizumab or disease progression, whichever occurred earlier. Data from this trial were presented at the 2010 meeting of the European Society of Medical Oncology (ESMO). A total of 1528 women were randomized from 263 centers. Compared to the control arm, the hazard ratio for disease progression in the bevacizumab arm was 0.81 (95% CI: 0.70–0.94, $P < 0.0041$).

In the setting of recurrent ovarian cancer, of great importance will be the mature results of AURELIA trial [39] (so far open to accrual), that is investigating the association of bevacizumab with platinum compounds both in platinum sensitive, and in platinum resistant patients.

The timing of bevacizumab administration during platinum-based regimens is believed to be a crucial point in the design of efficacious therapy in patients with recurrent disease.

The two ongoing phase III trials GOG213 [40] and OCEANS [41] take in consideration such parameter. Both trials target patients with recurrent disease: the former plans the administration of carboplatin and paclitaxel with or without bevacizumab in platinum sensitive relapsed OC patients, while in the latter carboplatin and gemcitabine with or without bevacizumab in recurrent disease, respectively.

In conclusion, up to now data arising from phase III trials show benefits in terms of Disease Free Survival (DFS). The benefit of bevacizumab on OS requires to be better investigated.

4.2. Epidermal Growth Factor Receptor Targeted Therapy: Trastuzumab, Cetuximab and Pertuzumab

4.2.1. Epidermal Growth Factor Receptor (EGFR) Family. EGFR family is a receptor family composed of four structurally similar tyrosine kinase receptors, ErbB1/HER1 (commonly referred to as EGFR), ErbB2/HER2 (commonly referred to as HER2), ErbB3/HER3, and ErbB4/HER4 [42]. They are expressed on the apical surface of epithelial cells. After binding with its ligand, the EGFR undergoes dimerization followed by tyrosine autophosphorylation, leading to the activation of EGFR signaling. Activation of downstream signaling pathways is known to mediate a variety of cellular responses, including cancer cell proliferation, survival, motility, and invasion. Moreover, as these receptors are overexpressed in many solid tumors, they have been recognized as promising targets for cancer therapy. Several MoAbs against the extracellular domain of EGFRs have been developed with the peculiar ability to block signaling of the receptor upon binding. Trastuzumab and Pertuzumab, directed against HER2 molecule and cetuximab (directed towards HER1), are in clinical use for several solid cancers. Also they have been evaluated in the framework of treatment regimens for ovarian cancer.

4.2.2. Trastuzumab (Anti-HER2). Trastuzumab is a humanized MoAb specific for the extracellular domain of HER2 that has been selected for its ability to block HER2 signaling after binding to the receptor HER2 is overexpressed in approximately 30% of breast cancers and is associated with a more severe prognosis [43, 44]. Trastuzumab is currently approved for refractory breast cancers positive HER2/neu either as a single agent or in combination with paclitaxel. To evaluate the therapeutic potential of trastuzumab in ovarian cancer, several preclinical studies have been conducted using HER2-expressing ovarian cancer cells [45, 46].

At least five potential extracellular and intracellular antitumor mechanisms of trastuzumab have been identified in the preclinical setting. These include activation of antibody-dependent cellular cytotoxicity, inhibition of the activatory extracellular domain cleavage, abrogation of intracellular signaling, reduction of angiogenesis, and decreased DNA repair [47]. Recently, also cellular adaptive immune system has been proposed to play a crucial role in trastuzumab clinical efficacy [48]. The overall results of these synergistic effects lead to tumor cell stasis and/or death.

On the basis of the promising results obtained in breast cancer patients [49] and the results of preclinical studies in ovarian cancer models [45, 46], the first phase II study evaluating the efficacy of trastuzumab in patients with recurrent ovarian cancers overexpressing HER2 was carried out in 2003 [50]. Forty-one women affected by recurrent or refractory ovarian or primary peritoneal carcinoma with 2+ or 3+ HER2 overexpression were enrolled. Patients without progressive disease or grade 3-4 toxicities could continue the treatment indefinitely. Patients with stable or responding disease were offered, after 8 weeks of treatment, to increase the weekly dose up to 4 mg/kg until disease progression. Median treatment duration was 8 weeks (range 2 to 104 weeks) and median progression-free interval was 2 months. Patients were analysed for the presence of soluble extracellular domain of HER2 and antibodies against trastuzumab. Circulating extracellular domain of HER2 increased during treatment in 8 of 24 evaluable patients. This immunological outcome was not associated to clinical outcome. No increase of anti-trastuzumab antibodies was observed. Although trastuzumab was well tolerated with common side effects of anemia, gastrointestinal disturbance, neuropathy, and fatigue, the overall response rate in these patients was only 7% with a median progression-free interval of 2 months. Interestingly, among patients with recurrent ovarian cancer who were screened for participation in the trial, only 11.4% were judged to have overexpression of HER2. On the basis of these results, the GOG was unable to recommend trastuzumab in OC.

4.2.3. Pertuzumab (Anti-HER2). Pertuzumab is a recombinant, humanized monoclonal antibody binding to the HER2 dimerization domain, sterically blocking the binding pocket required for receptor dimerization with its partner receptors, thus inhibiting the signaling cascades [51]. Pertuzumab binding to HER2 induces activation of ADCC effects but does not block the truncation of HER2 in the same way as trastuzumab binding does [52]. In a phase II study of 123

Table 2: Ongoing treatment studies evaluating MoAb treatment in ovarian cancer patients.

Protocol number	Disease stage	Target therapy	Treatment	Study phase	PI
NCT00565851 (GOG-213)	Recurrent	Bevacizumab	Arm I: Carboplatin + Paclitaxel/Docetaxel every 3 weeks Arm II: Carboplatin + Paclitaxel/Docetaxel + Bevacizumab every 3 weeks	Phase III	Coleman RL, MD
NCT00483782 (ICON 7)	Primary	Bevacizumab	Arm I: Carboplatin + Paclitaxel for 6 cycles Arm II: Carboplatin + Paclitaxel + Bev for 6 cycles + Bev for 12 cycles	Phase III	Perren TJ, MD
NCT00849667	Recurrent	Farletuzumab	Arm I: Carboplatin + Taxane + Farletuzumab 1.25 mg/kg Arm II: Carboplatin + Taxane + Farletuzumab 2.5 mg/kg Arm III: Carboplatin + Taxane + Placebo	Phase III	Morphotek, Inc
NCT00951496	Primary	Bevacizumab	Arm I: Carboplatin + Paclitaxel + bevacizumab for 6 cycles + bev until progression/recurrence Arm II: Carboplatin + Paclitaxel + bevacizumab for 6 cycles Arm III: Carboplatin + Paclitaxel + bevacizumab for 6 cycles	Phase III	Joan L. Walker, MD
NCT00976911	Primary	Bevacizumab	Arm I: Topotecan + Paclitaxel + liposomal doxorubicin Arm II: Bevacizumab + Topotecan + Paclitaxel + liposomal doxorubicin	Phase III	Hoffmann-La Roche
NCT01081262	Primary	Bevacizumab	bevacizumab capecitabine carboplatin oxaliplatin paclitaxel Procedure: quality-of-life assessment	Phase III	Martin E. Gore, MD
NCT01167712	Primary	Bevacizumab	Arm I: Carboplatin + Paclitaxel Arm II: Carboplatin + Paclitaxel	Phase III	John K. Chan, MD
NCT01239732	Primary	Bevacizumab	Arm I: Carboplatin + Bevacizumab + Paclitaxel	Phase III	Hoffmann-La Roche

recurrent ovarian cancer patients, 55 patients in cohort 1 and 62 in cohort 2 were evaluable for efficacy [53]. The patients in cohort 1 received a loading dose of 840 mg of pertuzumab intravenously followed by 420 mg every 3 wk; the patients in cohort 2 received 1050 mg every 3 wk and showed an overall response rate of 4.3%. The main adverse events observed were diarrhea and asymptomatic left ventricular ejection fraction decreases of <50%.

Combination therapy of pertuzumab with gemcitabine was tested in a randomized phase II trial in 130 patients with

Table 3: Ongoing treatment studies evaluating EGFR MoAbs in cervical cancer patients.

Protocol number	Disease stage	Target therapy	Adjuvant treatment	Study phase	PI
NCT00803062	Stage IVb/ recurrent/persistent	Bevacizumab	Cisplatin/topotecan hydrochloride/paclitaxel	Phase III	Krishnansu Tewari, MD
NCT00548418	Recurrent/Persistent	Bevacizumab	Topotecan/cisplatin	Phase II	Janet S Rader, MD

Table 4: Ongoing treatment studies evaluating VEGF MoAbs in cervical cancer patients.

Protocol number	Disease stage	Target therapy	Adjuvant treatment	Study phase	PI
NCT00292955	locally advanced/metastatic	Cetuximab	Cisplatin + radiotherapy	Phase II	Linda R. Duska, M.D
NCT00104910	Stages Ib-IVA	Cetuximab	Cisplatin + radiotherapy + brachitherapy	Phase I	John H. Farley, MD
NCT00997009	Advanced/Recurrent	Cetuximab	Paclitaxel + carboplatin	Phase II	Sandro Pignata, MD
NCT00957411	Stages IB-IIIB	Cetuximab	Cisplatin	Phase II	Susan Scholl, MD
NCT01158248	Stages Ib-III	Panitumumab	Cisplatin + Radiotherapy + brachitherapy	Phase II	Alain Zeimet
NCT01301612	Adenocarcinoma	Nimotuzumab	Cisplatin + Radiotherapy + brachitherapy	Phase II	Sergio Lago

platinum-resistant ovarian, fallopian tube, or primary peritoneal cancer [54]. The patients were randomly assigned to gemcitabine (800 mg/m^2 on days 1 and 8 of a 21-day cycle) plus either placebo or pertuzumab (840 mg loading dose followed by 420 mg every 3 wks) and showed objective response rates of 13.8% and 4.6%, respectively. Therefore, pertuzumab was able to significantly increase the effect of gemcitabine.

4.2.4. Cetuximab (Anti-HER1). Cetuximab is a chimeric MoAb that binds to the extracellular domain of EGFR (HER1).

It was developed to target the EGFR, thus preventing ligand activation of EGFR [55, 56]. In preclinical studies, cetuximab has been able to repress the growth of cultured A431 tumour cells and xenografts that expressed high levels of EGFR [57, 58]. In other solid tumours, cetuximab has shown to enhance the effects of different chemotherapeutic agents, including platinum [59, 60].

Based on these data, cetuximab was administered in combination with carboplatin to 28 patients with relapsed platinum-sensitive ovarian cancer. Cetuximab was infused at an initial dose of 400 mg/m^2 on cycle 1, day 1, followed by weekly infusions of 250 mg/m^2. Carboplatin (AUC 6) was administered IV on day 1 at 3-week intervals. The treatment was continued until disease progression or prohibiting toxicities. Twenty-six (92.9%) out of 28 patients were found to have EGFR+ tumours, whereas the remnant 2 patients (7.1%) had EGFR-tumours. Clinical response was reported for EGFR+ tumours: in 9 patients (34.6%) a clinical response was observed (3 (11.5%) Complete Response (CR); 6 (23%) Partial Response (PR)). Three patients (11.5%) had a progressive disease and the remaining 8 patients (30.8%) showed stable disease. The median PFS was over 9.4 months. Some grade 3 and three grade 4 toxicities were experienced but only three could be attributed to cetuximab.

In the same year, cetuximab combined with paclitaxel plus carboplatin was experimented as initial treatment in 40 advanced-stage ovarian, primary peritoneal, or fallopian tube cancer patients [61]. Thirty-eight out of the 40 participants had previously undergone abdominal surgery, whereas the remaining two patients were approached with neoadjuvant chemotherapy. The administration schedule consisted in an initial dose of cetuximab 400 mg/m^2 IV, followed by weekly infusions of cetuximab 250 mg/m^2, plus Paclitaxel 175 mg/m^2 and carboplatin (AUC 6) administered IV at 3-week intervals. Patients obtaining a complete clinical response after 6 cycles were eligible for a maintenance treatment with weekly cetuximab, for 6 months or until progressive disease or major toxicity. Thirty out of 40 patients completed all six cycles of chemotherapy and were evaluable for response: 21 of them achieved a complete clinical response. Twenty patients entered the cetuximab maintenance phase, but only ten completed all six cycles of cetuximab. Ten patients discontinued because of toxicity (5), progressive disease (2), grade 3-sinusitis (1), fluid accumulation (1), or other (1). The overall median time of PFS in the initial population was 14.4 months, with a third of the population with progressive disease after 24 months. Eleven (27.5%) out of 40 patients experienced at least one adverse event to cetuximab, with one case of grade 3-4 toxicity, whereas seven patients (17.5%) experienced toxicity to paclitaxel (three grade 3-4 toxicity). Consequently, although the combination of cetuximab, paclitaxel, and

Table 5: Ongoing treatment studies evaluating MoAbs in endometrial cancer patients.

Protocol number	Disease stage	Target therapy	Adjuvant treatment	Study phase	PI
NCT01010126	Endometrial cancer	Bevacizumab	Temsirolimus	Phase II	Charles Erlichman, MD
NCT00977574	Endometrial cancer	Bevacizumab	Temsirolimus Carboplatin ixabepilone Paclitaxel temsirolimus	Phase II	Carol Aghajanian, MD
NCT01005329	Endometrial cancer	Bevacizumab	Carboplatin Cisplatin Paclitaxel radiotherapy	Phase II	Akila Viswanathan, MD
NCT01367002	Uterine serous	Trastuzumab	Carboplatin Paclitaxel	Phase II	Alessandro D Santin, M.D.
NCT01256268	Endometrial Cancer	Ridaforolimus	Paclitaxel Carboplatin	Phase I	Robert Wenham, M.D.
NCR01244438	Endometrial Cancer with FGFR mutation	FP-1039	/	Phase II	Sarah Thayer
NCT01065246	Epithelial Carcinomas	Catumaxomab		Phase II	Jalid Sehouli, MD

carboplatin was well tolerated in this patient population, this study showed that this combination therapy failed to demonstrate a prolongation of PFS when compared with historical data. In a GOG phase II trial, single agent cetuximab demonstrated only minimal activity in patients with recurrent ovarian cancer with a response rate of 6.3% [62]. GOG has also evaluated the efficacy of cetuximab in the setting of combination therapy with carboplatin in patients with platinum-sensitive recurrent ovarian cancer. Results of this trial showed only modest activity with a response rate of 34.5% [56].

Considering these results, further efforts need to be carried out in the direction of identifying markers that can predict response before cetuximab can become point of the standard treatment.

4.3. EpCAM-Targeted Therapy: Catumaxomab. Catumaxomab is the first drug to be approved specifically for the treatment of malignant ascites, thus becoming one of the most successful monoclonal antibody to be employed in oncology. The approval dates back to April 2009, when the European Commission followed the recommendation of the Committee for Human Medicinal Products (CHMP) and approved catumaxomab for the i.p. treatment of malignant ascites in patients with EpCAM+ carcinomas resistant to standard treatments.

Catumaxomab (anti-EpCAM and anti-CD3) is a trifunctional monoclonal antibody with two different specificities, which binds simultaneously to the EpCAM on tumour cells and the CD3-antigen on T-cells. In addition, its Fc region composed by the two Ig isotypes mouse IgG_{2a} and rat IgG_{2b} selectively binds to human FcγI and III-receptors on innate immune cells, such as macrophages, dendritic cells, and NKs [63, 64]. Ertumaxomab (anti-HER2 x anti-CD3) is another trifunctional monoclonal antibody differing from catumaxomab only because it binds to HER2 rather than EpCAM [65].

Catumaxomab and ertumaxomab were firstly administered intraperitoneally to eight patients with malignant ascites (two of which with ovarian cancer) with the aim of verifying their tolerability and biological and clinical effects [29]. The two ovarian cancer patients were treated with both catumaxomab and ertumaxomab at different administration schedule. The first ovarian cancer patient received six administrations (five with catumaxomab and two with ertumaxomab) during a 13-day period, whereas the other patients were treated with five immunizations (five with ertumaxomab only and the last one in combination with catumaxomab). The treatment was well tolerated by all the eight patients enrolled. Resolution of ascites was experienced by all participants and seven out of eight participants did not require further paracentesis during the follow-up, with a median ascites-free interval of 38 weeks. As expected, the resolution of ascites was correlated with elimination of tumour cells ($P < 0.0014$) as detected by FACS analysis and immunocytochemistry. Complete elimination of EpCAM and HER-2/neu tumour cells in ascites was obtained for both ovarian cancer patients, that succumbed after 22 and 41 weeks, respectively.

Catumaxomab was also tested in a phase I/II dose-escalating study on 23 women affected by advanced ovarian cancer with symptomatic malignant ascites containing EpCAM+ tumour cells [63]. The participants were divided into six different groups and treated with four to five intraperitoneal catumaxomab in dose 5 to 200 μg on days 0, 3, 6, 9, and 13. All patients were evaluated for toxicity, clinical response, and immunological status. Serious adverse events were detected in 15 out of 23 patients. In six patients they were considered treatment-related. In the majority of participants, a significant decrease of ascites flow rate was

observed after the third infusion, compared to baseline. Twenty-two out of 23 patients did not require further paracentesis after the last infusion, until the end of the study at day 37. The results of the prospective randomized phase II/III study published by Heiss et al. in 2010 [66] confirmed the efficacy of catumaxomab in the management of malignant ascites. Two hundred and fifty-eight patients affected by EpCAM+ epithelial tumor-related malignant ascites (129 recurrent ovarian cancer) were randomly assigned to receive paracentesis followed by four i.p. infusion of catumaxomab, in a ten-day period, or paracentesis alone. The Intention to-Treat (ITT) analysis revealed that puncture-free survival was significantly longer in the catumaxomab group than control group (46 versus 11 days; $P < 0.0001$), as well as the median time required for the next paracentesis (77 versus 13 days; $P < 0.0001$). A positive trend in OS was observed in the whole catumaxomab group and in the ovarian cancer patients catumaxomab subgroup; furthermore a significant increase in OS was observed in the gastric cancer patients catumaxomab subgroup. Catumaxomab-related adverse events were manageable, reversible, and associated to an acceptable safety profile.

However, positive results were not reached by catumaxomab treatment in terms of tumor response. In fact, the Phase IIa Study of the AGO Study Group revealed that catumaxomab has modest activity in platinum-resistant ovarian cancer, with only 5% of partial response being obtained with high-dose catumaxumab [67].

4.4. Folate Receptor Alpha-Targeted Therapy: Farletuzumab. Farletuzumab is a humanized MoAb with high affinity for folate receptor α (FRα). This receptor, almost absent in normal tissue, is overexpressed in most ovarian cancers, making it an attractive therapeutic target.

Preclinical studies have demonstrated that farletuzumab mediates robust antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity *in vitro*, inhibits tumor growth in ovarian tumor xenografts, and displays a safe toxicology profile in not human primates [68, 69].

Farletuzumab has shown clinical efficacy in early phase trials as single agent and combination therapy with minimal drug-specific toxicity [70].

The Phase III development plan in ovarian cancer patients includes combination chemotherapy studies in both platinum-sensitive (recently launched) and platinum-resistant (planned) recurrent disease.

4.5. CA125-Targeted Therapy: Oregovomab. CA125 is a surface mucin-like glycoprotein antigen that is expressed in more than 95% of all not mucinous stage III/IV epithelial ovarian cancers (EOCs) [71].

Serum CA125 level is a highly useful and well-established surrogate for monitoring the response to treatment and a useful marker during follow-up [72, 73].

Oregovomab is a MoAb against the tumour-associated antigen CA125 as both membrane bound and soluble forms. Oregovomab administration induces both cellular and humoral multiepitope immune responses against the tumor cells [74]. Based on the observation that ovarian cancer patients, injected with this agent for diagnostic purpose, showed prolonged survival [75], in 1998 the immunological effects of oregovomab were tested in 75 ovarian cancer patients [76]. All participants received from one to ten injections of the MoAb and, after vaccinations, 64% of them developed anti-idiotypic antibodies against oregovomab, whereas 24% developed anti-CA125 antibodies. It was observed that these two types of antibody were able to induce Fc-mediated tumour cell killing. Moreover, a higher significant survival was observed in patients in which anti-CA125 antibody concentration increased more than 3-fold after oregovomab administrations, compared to patients without such increase. Furthermore, an improved overall survival was observed also in patients who developed specific anti-CA125 B- and T-cell response after oregovomab administration [77]. In 2004, Gordon et al. [78] vaccinated women suffering from recurrent ovarian cancer with oregovomab. Significant increases in T-cell responses were measured in 7/18 (39%) patients in response to CA125, in 5/8 (63%) patients in response to autologous tumor cells, and in 9/18 (50%) patients in response to oregovomab. Immune responses appeared by week 12 (four doses) and were generally maintained or augmented in patients maintaining combined treatment with oregovomab and chemotherapy. Median survival was 70.4 weeks (4.6–141.6 weeks), and the median progression-free interval was 11 weeks (2.6–114.6 weeks). Patients who mounted a T-cell response to CA125 and/or autologous tumor showed significantly improved survival compared to patients who did not.

In 2004, Berek et al. [79] enrolled 145 patients affected by advanced ovarian cancer (FIGO stage III-IV) in a randomized placebo-controlled study, to assess safety, feasibility, and toxicity of oregovomab administration and to evaluate this reagent as consolidation treatment. Unfortunately, despite a benign safety profile, Time to Relapse (TTR) was not significantly improved by consolidation therapy with oregovomab (13.3 months oregovomab versus 10.3 months for placebo; $P = 0.71$).

One year later, Ehlen et al. [80] showed immune and clinical results of a pilot phase 2 study concerning oregovomab-based vaccination in 13 patients with recurrent ovarian cancer. Immune responses, including antibodies and T cells to oregovomab and CA125, were demonstrated in more than half of the patients. Disease stabilization and survival >2 years was observed in 3 of 13 patients and coincided with robust immune responses. Shrinkage of marker lesions was not observed; however, four patients showed decreases in CA125 levels. Treatment was well tolerated without serious adverse events. This pilot study supported immunologic activity and safety of oregovomab in recurrent OC.

Long-term clinical results of this study were showed in 2008 [81], after a 5-year follow-up. Patients assigned to the oregovomab and placebo groups lived a median of 57 and 48.6 months of progression-free survival ($P = 0.276$), respectively. Considering the time of survival after relapse, oregovomab and placebo groups lived 31.2 and 20.7 months, respectively. Further analyses from this study were recently reported [82]. A total of 371 patients were

included in the study: 251 were treated with oregovomab and 120 were assigned to the placebo group. After five years, 169 and 80 patients belonging to the oregovomab and placebo group, respectively, were still on treatment. It was observed that the median time to relapse was 10.3 months for oregovomab and 12.9 months for placebo-treated patients, respectively. Survival data were not available at the time of the report. The incidence of treatment adverse events was similar in both groups. These data indicate that patients with advanced ovarian cancer do not benefit from oregovomab maintenance monoimmunotherpy.

In 2009, a study assessing this immunotherapy at 2 dosing schedules in 40 patients with advanced ovarian cancer undergoing front-line carboplatin-paclitaxel chemotherapy showed that combination of oregovomab immunotherapy and chemotherapy exerted immune adjuvant properties. The possible combination of carboplatin and paclitaxel chemotherapy with oregovomab and other antigen-specific cancer immunotherapy approaches should be further investigated.

Results obtained so far indicate that although CA125 remains an attractive target for immunotherapy, no effective clinical benefit was observed by targeting this mucin.

4.6. MUC1-Targeted Therapy. MUC1 is a heavily glycosylated transmembrane glycoprotein that is overexpressed in many carcinomas [83, 84]. MUC1 consists of three domains (a large extracellular motif, a transmembrane motif, and a cytoplasmic tail) [85] and mediates signal transduction events that stimulate the motility, invasion, and metastasis of cancer cells. MUC1 is overexpressed on 90% of early ovarian cancer cell surfaces [83]. In cancer patients, humoral and cellular responses against MUC1 have been detected [86, 87]. Thus, MUC1 has been recognized as a promising molecular target for immunotherapy in patients with ovarian cancer.

The mAb Human Milk Fat Globule 1 (HMFG1) is a murine MoAb that recognizes an epitope localized in the extracellular MUC1 domain.

In a first phase I/II study, Yttrium-90-labeled HMFG1 alone or in combination with Yttrium-90-labeled MoAb AUA1 (directed against an unspecified ovarian cell surface antigen) was intraperitoneally administered to 25 patients with advanced ovarian cancer, who previously had undergone cytoreductive surgery followed by chemotherapy [88]. Fourteen patients had assessable tumour at laparoscopy; none of the three patients with tumour nodules greater than 2 centimetres diameter showed any response to treatment, although one patients experienced resolution of her ascites. One out of ten patients with tumour nodules less than 2 centimetres diameter had a partial response which persisted for one year. Most frequent toxicities consisted in reversible myelosuppression and thrombocytopenia.

An extended study [89] comparing radioimmunotherapy (90Y-labeled HMFG1) after chemotherapy with chemotherapy alone in 45 ovarian cancer patients, disease-free at second-look laparoscopy, found that the active arm had a significantly higher OS (80%) at five-year follow-up, as compared to the control group (50%).

Ten years later, a clinical study [90] carried out on 52 ovarian cancer patients (40% in complete clinical remission and 60% with residual disease) revealed that a single intraperitoneal administration of ^{90}Y-radiolabeled HMFG1 could prolong long-term survival, with a 10-year survival rate of 70%.

In 2004, 26 women affected by ovarian cancer received a priming dose of 25 mg of HMFG1 either intravenously ($n = 10$) or intraperitoneally ($n = 16$), followed by 6 intradermal immunizations of HMFG1 in 10% Alhydrogel at 1-month intervals [91]. The 3 dose levels were 0.5 mg, 1 mg, and 5 mg. Thirteen out of 26 patients completed the treatment, while the other patients had clinical disease progression.

ELISA showed that all patients generated measurable anti-idiotypic Ab (Ab2) after 3 immunizations, sustained at 1 month after the final booster. No statistically significant differences were observed in the levels of Ab2 generated by higher or lower booster doses or between patients whose first immunization was administered intravenously or intraperitoneally. 5/13 patients (38%) increased anti-MUC1 levels above 0.015 μg/mL (pretreatment peak). Ab3 (anti-anti-idiotypic Ab) changes for the group as a whole were not statistically significant ($P = 0.065$). Furthermore, anti-MUC1 levels did not correlate with Ab2 levels. Biosensor assay, using the resonant mirror biosensor, showed no difference in the affinity of Ab2 generated by different booster doses of HMFG1. No clinical response was detected in patients with measurable disease, although 1 patient remained without clinical disease for 5 years after completion of vaccination.

The major study investigating safety and efficacy of Y-90-labeled HMFG1 [92] was carried out on 447 women with FIGO stage IC to IV epithelial ovarian cancer in complete remission of disease, surgically assessed through a second-look laparoscopy. In this randomized control study, 224 patients were assigned to receive standard treatment plus a single intraperitoneal infusion of 25 mg Y-90-labeled HMFG1, whereas 223 patients received standard treatment alone. Clinical results showed that there was no significant difference in terms of OS ($P = 0.4033$) and PFS ($P = 0.4764$) between both groups, concordantly with time to serological relapse (CA125 increase) ($P = 0.3140$). However, it was observed [93] that significantly fewer intraperitoneal ($P < 0.05$) and more extraperitoneal ($P < 0.05$) relapses occurred in patients who received Y-90-labeled HMFG1. Furthermore, time to IP recurrence was significantly ($P = 0.0019$) longer and time to extraperitoneal recurrence was significantly shorter ($P < 0.001$) for the active treatment arm. Although serious adverse events occurred with no significant differences in both two arms, hematologic toxicities were more frequent in the Y-90-labeled HMFG1, with a peak incidence after the sixth week of treatment. Serum sample from 208 patients in the active treatment group and 199 patients in the standard treatment group were evaluated for anti-MUC1 IgG [94]. Anti-MUC1 IgG titers ranked significantly ($P < 0.001$) higher in the active treatment group when tested at weeks 4, 8, and 12. A significant difference ($P < 0.001$) in terms of median area under the curve (AUC) between both groups was observed in favour of the active treatment group. A significant higher benefit in OS and disease-free survival ($P = 0.043$ and 0.036, resp.) for patients

of the active treatment group with an anti-MUC1 IgG AUC > 13 was shown by multivariate analysis and Kaplan-Mayer analysis.

Recently, a humanized variant of the murine HMFG1, AS1402, has been developed and is now being studied in a phase II trial evaluating the efficacy of the combination of AS1402 with hormonal therapy in postmenopausal women with advanced breast cancer [95]. This humanized antibody could represent a potential treatment agent for patients with ovarian cancer.

4.7. Targeting Immunesuppressive CTLA-4: Ipilimumab. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a surface ligand expressed by activated lymphocytes, which binds to B7-1 and B7-2 ligand expressed upon APC membrane for cell-cycle arrest and attenuation of effector function. Consequently, CTLA-4 acts as a negative regulator of immune response. Ipilimumab is a humanized monoclonal antibody blocking CTLA-4, engineered to contrast the negative immune regulation, thus increasing quantity and duration of the immune effector response against tumor cells. Very recently, the results of significant improvement in overall survival obtained in melanoma setting [96] have led the FDA to approve ipilimumab in the treatment of metastatic melanoma disease (http://www.fda.gov/, FDA approval in August 2010), making the emergence of the key role of CTLA-4 as a therapeutic target in oncologic patients possible. Up to now, only one experience has been carried out with ipilimumab in ovarian cancer.

In 2008 Hodi et al. [96] administered 1 to 11 infusions ipilumimab in 9 patients with stage IV ovarian cancer and in 11 patients with metastatic melanoma. Each dose (3.0 mg/kg over 90 minutes) was administered at 2-3 months interval, with the exception of 1 ovarian cancer who was treated at 3- to 6-month intervals. Eligibility criteria included previous vaccinations with irradiated, autologous tumor cells engineered to secrete GM-CSF (GVAX). The most relevant results involved 1 patient, who achieved a significant fall of CA125 levels several months after the first dose of ipilumimab. The antitumoral response did not involve the generation of anti-CA125 Ab, but the increase in humoral response against NY-ESO-1 was associated with therapeutic effects.

5. Monoclonal Antibodies in Cervical Cancer

Currently no treatment with MoAbs has been authorized by FDA for patients with cervical and endometrial malignancies and therefore only experimental results are available.

In cervical cancer patients two molecules are currently investigated as target for MoAbs-specific treatment: VEGF and EGFR.

5.1. Vascular Endothelial Growth Factor. In cervical cancer patients VEGF overexpression is associated with tumor progression and poor prognosis [97, 98]; higher VEGF levels appear to correlate with a more advanced disease stage and increased risk of lymph nodes metastasis [99].

Furthermore, it has been shown that higher VEGF expression, as well as increased tumor vascularization, is independent predictors of poor disease and OS [100].

Several studies performed on bevacizumab, used as single agent or in association with other drugs, demonstrated that this antibody is able to delay the progression of cervical cancer [101, 102]. All patients received chemoradiation and at least one other chemotherapy regimen prior to this combination therapy with bevacizumab. After treatment, one patient achieved complete response, one partial response, and two showed disease stabilizations. Furthermore we have encouraging results on phase II multicenter trial to assess the efficacy and tolerability of bevacizumab used as single agent in patients with persistent or recurrent squamous cervical carcinoma.

Approximately, 24% of women benefited from a progression free over 6 months and in 11% of patients a partial response was observed [101]. The only phase III trial with MoAb in cervical cancer is ongoing now [103] (Table 4). Results of this phase III trial are expected both for bevacizumab alone and in combination with chemotherapeutic agents in recurrent/persistent or, mostly, stage IV cervical cancer. In IV stage cervical cancer patients, a real standard of care has not been well established nowadays [104]. Surgery, when technically feasible, intuitively appears as the most direct way to eliminate tumour burden and overcome radio and chemotherapy resistance caused by size in this stage of disease. Unfortunately, several patients affected by large volume disease are considered inoperable. Neoadjuvant chemotherapy (NACT) has demonstrated to increase the proportion of women amenable of surgery and reduce negative pathologic prognostic factors [105, 106]. Severe prognosis is associated with this stage and the fact that these patients are affected by chemo-naive neoplasms makes this setting of women particularly adequate to test new combinations drug that include target therapies.

5.2. Epidermal Growth Factor Receptor. EGFR is tyrosine kinase receptor of the family that includes HER2, HER3, and HER4. This receptor mediates cell differentiation and proliferation in both embryonic and adult tissues.

This receptor is overexpressed in approximately 85% of invasive cervical tumours and is associated with higher stages and poor prognosis [107–111]. Blockage experiments of this receptor show that it exerts a positive modulation of adjuvant treatments. In particular, in human tumour xenograft *in vivo* model MoAbs showed synergistic effects with cisplatin and doxorubicin [112] and in human this effect was observed with radiotherapy [57].

Between MoAb directed against EGFR, the ones that are currently studied in cervical cancer patients are cetuximab and matuzumab.

Cetuximab (Table 3) is a chimeric IgG1 mAb that antagonizes normal ligand receptor interactions and therefore disrupts EGFR downstream signaling. The relation between EGFR protein expression and response to mAb is doubtful, as colorectal cancer patients without protein overexpression may respond to cetuximab [113].

Preclinical studies developed on cervical cancer cell lines [114, 115] confirm even in this tumor the results obtained in murine model from Baselga group: both chemo and radiotherapy join of cetuximab coadministration effects but apparently in a less EGFR-dependent way [115]. Less encouraging are clinical results. No PFS and OS benefits have been registered in cervical cancer patients in either advanced, recurrent, metastatic, or pretreated disease [116–119]. However, better outcomes could result from an ongoing clinical trial evaluating the addition of cetuximab to standard treatment in patients with early stages of cervical carcinoma.

6. Monoclonal Antibodies in Endometrial Cancer

In endometrial cancer patients only VEGF is currently investigated as target for MoAbs-specific treatment. Therefore, data on the possible role of Bevacizumab in endometrial cancer are still scarce [120, 121].

6.1. Vascular Endothelial Growth Factor. VEGF is critical for angiogenesis and tumor progression. Preliminary results from studies conducted with the purpose of evaluating the role of antiangiogenic agents in patients with endometrial cancer are encouraging [121]. A study [120] on recurrent or persistent endometrial cancer with bevacizumab showed 8/53 (15.1%) response rate, with 1 complete response and 7 partial responses. Median PFS was 4.2 months. Median OS was 10.5 months. Several clinical trials are currently ongoing (Table 5).

7. Discussion and Conclusion

Monoclonal antibodies have demonstrated to be effective in both hematologic and solid malignancies. This family of antineoplastic agents have several different mechanisms, such as binding soluble ligands, blocking cell receptors, and activating ADCC.

In ovarian cancer, encouraging results have been observed with bevacizumab in first and second line settings, mostly in association to standard chemotherapy regimens. Currently, the primary goal in combining bevacizumab to standard chemotherapy is to test its efficacy in increasing the duration of first remission. Preliminary results of randomised trials carried out with this purpose seem to confirm a benefit in terms of progression-free survival, whereas data regarding overall survival remain currently less clear. Furthermore, on the basis of the Japanese experience [122], the GOG 262 is now testing the association between bevacizumab and paclitaxel in a dose dense front line regimen. The rational of combining bevacizumab to weekly paclitaxel in first line setting dates back to recent evidences showing that this association significantly improves progression-free survival in heavily pretreated recurrent epithelial ovarian cancer [123, 124], thus confirming the role of weekly paclitaxel plus bevacizumab in synergistically inhibiting angiogenesis [125].

As single agent in ovarian cancer palliative setting, cetuximab remains one of the most successful monoclonal antibodies to be employed, with demonstrated and approved high efficacy in the management of ovarian cancer-related malignant ascites. Another promising antibody, in gynecologic oncology, seems to be farletuzumab, targeting the folate receptor which is widely expressed by ovarian cancer cells.

Despite the recognized clinical role of trastuzumab-based therapy in breast cancer, current evidence seems to deny any possible clinical relevance of single-agent trastuzumab-based treatment both in ovarian cancer and in endometrial cancer settings [126]. Studies assessing the association between trastuzumab and standard chemotherapy regiments in these types of gynecological malignancies are required.

Promising results have been currently obtained in ovarian cancer setting by single-agent HMFG1 administration, even if stratification of the results in terms of tumor histology would clarify the most appropriate subset which can mostly benefit from anti-MUC1 vaccination. Clinical evaluation of combining HMFG1 to chemotherapy is strongly needed.

Currently, no antibody has shown a particularly high activity in cervical neoplasm. The high expression rate of EGFR, targeted by cetuximab, makes this monoclonal antibody one of the most studied new drugs, even if some concern has been raised for the tolerability of this drug in previously irradiated patients. In ovarian cancer, this drug revealed no positive clinical benefit, both as single-agent and in association to standard chemotherapy regimens. Furthermore, its combination to chemotherapy seems to enhance the risk of treatment-associated adverse events, thus discouraging future employment of this monoclonal antibody in this setting.

In endometrial cancer some experience has been gained with bevacizumab. Results appear comparable to what observed with nontarget drugs.

Up to now, target therapies have not been investigated in rare gynecological malignancies such as vagina and vulvar cancer.

Target therapies and in particular monoclonal antibodies were introduced in oncology with the expectation of having extremely favorable side effects. On the contrary, hemotoxicity, dermotoxicity, gastrointestinal toxicity, and high rates of thromboembolic events have all been reported. With the exception of bevacizumab, no target therapy has yet shown a clear therapeutic effect in gynecological malignancies.

Target therapies are in their infancy in gynecologic oncology. The magnitude of their clinical impact is yet to be seen. A crucial point that requires further investigations remains to be patient selection and targets identification.

MoAbs are destined to become an important tool in the hands of oncologists that treat neoplasms of the genital tract but, as all other established treatments, they will carry the burden of a learning curve to manage their new side effects.

Conflict of Interests

All named authors declare that there are no conflict of interests.

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Research Article

Gender-Specific Cytokine Pathways, Targets, and Biomarkers for the Switch from Health to Adenoma and Colorectal Cancer

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Studies focusing on gender have shown that differences exist in how the immune system responds to disease and therapy. Understanding how gender influences immunological mechanisms in health and disease and identifying gender-specific biomarkers could lead to specifically tailored treatment and ultimately improve therapeutic success rates. T helper1 (Th1) and Th2 cytokines (Th1/Th2) have pivotal roles in the homeostasis of Th1 and Th2 cell network functions in the immune response but sex steroids affect Th1/Th2 production in different ways and a natural sexual dimorphism in the immune response has been shown. In order to investigate these differences further, we developed Th-cytokine data-driven models of the immune response and evaluated healthy subject peripheral blood samples. Independent cohorts of colorectal cancer and adenoma patients were also studied for comparison purposes. Our results show that the interferon (IFN)γ production pathway for immune response homeostasis is specific to men whilst the interleukin- (IL-) 6 production pathway for immune response homeostasis is specific to women. The IL-10 pathway for restoring immune system resting homeostasis was common to both but was controlled by the respective gender-specific pathways. These gender pathways could well be used as targets and biomarkers in translational research into developing new clinical strategies.

1. Introduction

Advances in the understanding of pathological mechanisms and the identification of disease targets and biomarkers have had a considerable impact on clinical practice [1]. One change has been the shift from generalized medicine to a stratified approach, with patients being placed in clinical diagnostic or therapeutic subgroups according to specific biomarkers [2, 3]. It is hoped that this approach will lead to more specific and effective treatment in the not too distant future but this success depends upon the identification of specific biomarkers that can be measured easily from disease onset. Peripheral blood targets/biomarkers are currently the most practical, noninvasive means of diagnosing disease, predicting prognosis, and therapeutic response [4]. The identification of gender-specific biomarkers in peripheral blood would therefore open up an interesting field for research given gender-related susceptibility to disease [5]. Sex steroids, for example, have been shown to influence the regulation of Th cell network balance, shifting the balance toward a Th1 and/or Th2 type response, and both clinical and experimental data have demonstrated the presence of a natural sexual dimorphism in the immune response [5–8]. During their reproductive years, females have a more vigorous cellular and humoral immune response than males and a greater ability to reject tumors and homografts [9–14]. Evidence suggests that physiological levels of estrogen affect humoral and cell-mediated immune responses, while the male hormone, testosterone, does the opposite [15–17]. Ironically, this enhanced baseline immune function is associated with a higher prevalence of autoimmune disorders in females of reproductive age [6], than in postmenopausal women or men [18–21]. Sex steroids seem to affect Th1/Th2 production in different ways: during pregnancy, the Th1/Th2 network balance is skewed toward Th2 [22], thereby preventing rejection of the antigenically foreign fetus by a cell-mediated

immune attack [23–26]. The *in vitro* influence of sex steroids on T-cell cytokine production has been studied extensively [27–30], showing, however, complex and diverse effects.

We believe that differences in Th1/Th2 production pathways in men and women are responsible for differences in the immune response in health and disease. Gender differences in immunological pathways imply different reactions to disease as well as different reactions to drugs and hence the identification of these gender-specific pathways could lead to more successful treatment.

In order to demonstrate these differences, we developed Th-cytokine data-driven models of the immune response and evaluated peripheral blood samples taken from healthy men and women. Independent cohorts of colorectal cancer and adenoma patients were also evaluated for comparison purposes. Our study indicates, for the first time, that gender-specific Th1/Th2 pathways operate in maintaining the homeostasis of the immunological cell network. These gender-specific pathways may well be responsible for differing gender-dependent responses to disease and therapy and open up an exciting new field for research.

2. Materials and Methods

2.1. Experimental Design. Human studies were performed in accordance with the standards of the Ethics Committee and all persons gave their informed consent prior to their inclusion in the study. To establish whether gender-specific Th1/Th2 cytokine production pathways could be at the basis of differences in immunological responses we designed an experimental approach based on the use of cytokine data-driven computational models of the immune response (Figure 4).

Whole blood levels of Th1 and Th2 cytokines, indicative of Th1 or Th2 cell differentiation, were used (Figure 4); the relative proportion of each Th cell-type generation depends on the cytokines produced by APCs (cellular network) and released into the cell environment during resting and activation states of the immune response. Whole blood contains all blood cells and the cell environment and so it includes the cytokine levels from the "cellular network" and the "environment network", reflecting *in vivo* physiological conditions more accurately and so appropriate to this study.

We worked on the assumption that the network profile of the production levels of Th1 and Th2 cytokines (*level network profile*) reflected Th1 or Th2 differentiation: balance between the levels of Th1 and Th2 cytokine production indicated normal Th1 and Th2 cell differentiation and so a productive immune response.

We determined level network profiles in whole blood culture supernatants without activation (APC and T cells in resting conditions) and with LPS (activated APCs) and PHA (activated T cells). We also analyzed (i) the PHA-level network profiles of separated (Ficoll/Hypaque gradient) peripheral blood mononuclear cells (PBMCs), in order to discover whether T cellular components affect Th1/Th2 interaction; and (ii) the level network profiles in blood serum, to identify gender-specific Th1/Th2 biomarkers. The level network profiles were also determined in colorectal cancer and colon adenoma patient groups divided by sex, as independent cohorts for comparison purposes.

The cytokines used in our Th-cytokine data-driven computational models of the immune response were as follows: IL-2, IFNγ, IL-4, IL-6, and IL-10, to make up our basic network model, to establish if the direction in T cell differentiation was Th1 and/or Th2 type; tumor necrosis factor (TNF) α and IL-1β as serum biomarkers and IFNγ, IL-6, and IL-10 as LPS whole blood biomarkers, to determine if antigen presenting cells (APCs) direction on T cell differentiation was of Th1 or Th2 type; and soluble (s) IL-2 receptor (R) and sIL-6R to estimate cell activation. Indeed, IL-2 and IFNy support Th1 functions [31] promoting cell-mediated immunity; IL-4, IL-6, and IL-10 are associated with Th2 responses and IL-10 is a powerful inhibitor of IFNγ and macrophages [32]. IL-6 also supports Th17 functions, suppressing Th1 function [33, 34], and has a key function in homeostasis influencing Th differentiation into T regulatory (Treg) or Th17 cell subsets. TNFα and IL-1β, on the other hand, are some of the key mediators produced by APCs that dictate the course of immune responses. sIL-2R and sIL-6R are activation markers [35, 36].

2.2. Healthy Subjects. A group of 66 healthy subjects were studied (33 men and 33 women). None of the subjects were receiving concurrent drug treatment including widely used pharmaceuticals, such as salicylates and sex hormones (contraceptive pill, hormone replacement therapy). Distribution of age in the male and female groups was the same (men: $N = 33$ mean $\pm$ SD = 41 $\pm$ 12.00 years; women: $N = 33$ mean $\pm$ SD = 41 $\pm$ 15.00 years; $P = 0.14$).

2.3. Independent Validation Cohorts of Colorectal Cancer and Adenoma Patients. A group of 110 patients, 64 men and 46 women, who were diagnosed for the first time as having colorectal cancer and had to undergo colectomy were studied. Distribution of age in male and female groups was the same (men: mean $\pm$ SD = 65.60 $\pm$ 10.90 years; women: mean $\pm$ SD = 65.90 $\pm$ 10.40 years; $P = 0.89$). Clinical diagnosis was confirmed histopathologically and patients were subtyped using the pTNM classification (according to the diagnostic criteria of the American Joint Committee on Cancer and the Committee of the International Union Against Cancer), as follows: men 16 stage I, 30 stage II, 8 stage III, 10 stage IV; and women 4 stage I, 23 stage II, 13 stage III, and 6 stage IV. None of the patients received radiation or chemotherapy before surgery. Distribution of stage in male and female groups was the same ($P = 0.87$). Tumors varied from 2.5 to 9.0 cm in diameter.

A group of 8 colon adenoma patients, 4 men and 4 women, were also studied. Distribution of age in the male and female groups was the same (men: mean $\pm$ SD = 64.75 $\pm$ 4.99 years; women mean $\pm$ SD = 69.50 $\pm$ 13.02 years, $P = 0.52$). Clinical diagnosis was confirmed histopathologically. Distribution of age in male and female groups between colorectal cancer and adenoma patients was also the same ($P = 0.61$).

2.4. Blood Samples. Blood was collected at the same time of day to minimize the effects of diurnal variation. A 15 mL sample of heparinized (Liquemin-Roche) blood (20 IU heparin/mL blood) was taken from each subject, and the samples, kept at room temperature, were used immediately in whole blood cell cultures. Additionally, 5 mL sample of peripheral blood without heparin was also taken and, within 1 h of withdrawal, the serum was stored in aliquots at −80°C until use.

2.5. Whole Blood Cell Cultures. Heparinized venous blood [37] was diluted 1 : 10 with RPMI-1640 medium (Sigma, endotoxin tested), which was supplemented with L-glutamine 0.2 mM, penicillin 50 IU/mL, and streptomycin 50 μg/mL (Sigma) and distributed in 0.5 mL aliquots in 12 mm polystyrol tubes. 10 μg/mL of PHA and 10 μg/mL of LPS (Sigma) were used for stimulation; aliquots without stimuli were also prepared. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. After 24 h and 72 h of culture without a change of medium, 320 μL supernatant was removed from each tube to be assayed for cytokine levels and stored in aliquots at −80°C until used. The effect of adding heparin which prevents clotting in whole blood cultures was tested (data not shown).

Experimental conditions were as follows: with stimuli (+PHA and +LPS) in order to recreate an activation situation and without stimuli to evaluate immune response in resting conditions. PHA was used to study T cell contribution [38] and LPS the influence of antigen presenting cells [39]. As mentioned in the "experimental design" the whole blood culture method was used [40]. It is a simple procedure and thus reduces the potential for error and offers the added advantage of not requiring the separation of cellular subpopulations which would represent an additional source of potential variation both in individuals and among individuals.

2.6. PBMC Cell Cultures. PBMCs were separated by centrifugation over a Ficoll/Hypaque gradient (20 min, 1000 ×g) and washed with RPMI-1640 medium (Gibco). Isolated cells were cultured at a concentration of 1×10^6 cells/mL in RPMI-1640 complete medium (supplemented with 10% fetal calf serum, L-glutamine 0.2 nM, penicillin 50 UI/mL, streptomycin 50 μg/mL; Sigma). Supernatants were obtained from PBMC cultures in RPMI-1640 complete medium. The cells (with and without PHA, 3 μg/mL) were incubated at concentrations of 1×10^6 cells/mL at 37°C in a humidified atmosphere of 5% CO_2. After 24 h of culture without a change of medium, 120 μL supernatant was removed from each well, centrifuged at 250x g, and stored in aliquots at −80°C until use.

2.7. Cytokine Detection. ELISA assays were used. This method has been described in detail elsewhere [41]. For intraassay precision, standard samples of known cytokine concentrations were assayed in replicates of 10, and the coefficient of variation was <10%. For interassay precision standard samples were assayed 30 times in multiple assays to determine precision between assays, and the coefficient of variation was <10%.

The sensitivity of these ELISA assays was as follows: sIL-2R <50 IU/mL, TNFα <1.5 pg/mL, (T Cell Diagnostics-Cambridge, USA), IL-1β, IL-2 <5 pg/mL, and IL-4 <1 pg/mL (Endogen, Cambridge, USA); sIL-6R <4, 3 ng/mL (Biosource, Belgium); IL-6 <2 pg/mL, IFNγ <4 pg/mL, and IL-10 <5 pg/mL, (Benfer-Scheller, Keystone, USA). Cytokine values were obtained using a specific software program (ELISA-AID, Eurogenetics).

2.8. Statistical Analyses. In physiological systems components operate as a network and individual network components vary dynamically and covary with respect to one another. Therefore, the identification of Th-cytokine physiological pathways in this study and correlated biomarkers can only be achieved through evaluations that take into account systems biology characteristics [42, 43]. This entails determining the level of cytokines, the study of the relationships between cytokine levels, and then the behaviour of this multicomponent system as a network. Due to the complexity of biological systems, this requires the use of mathematical models that provide a framework for determining the outcome of numerous and simultaneous time-dependent and space-dependent processes [44–46]. Hence, in addition to the study of statistical differences between Th1 and Th2 cytokines, using the Mann-Whitney U test or the Student's t-test (as appropriate), we studied data-driven Th1/Th2 cytokine models through multivariate statistical analyses using "Statgraphics software systems" (full system 5.25 version 4.0; graphics system by statistical graphics corporation ed., USA, 1989). Values of $P \leq 0.05$ were considered significant.

We used the multivariate statistical procedure that analyses the correlation between parameters and produces a matrix of correlation coefficients (that vary from −1 to +1) and significance (P), allowing a dynamic analysis of how network components vary with respect to one another at any moment in time. A positive correlation indicates that the parameters vary in the same direction, while negative correlation indicates that the parameters vary in the opposite direction. In fact, the multivariate statistical procedure that analyses the correlation measures the linear associations between all parameters, and if parameters increase or decrease at the same time, the correlation is positive, whilst other changes are considered negative. Statistically independent parameters have an expected correlation of zero.

The multiple regression analysis, which provides a modeling technique that allows us to relate a dependent variable to one or more independent variables, was also used. Regression analysis allows us to summarize data and quantify the nature and strength of the relationships among variables. Hence, the multiple regression and stepwise multiple regression analyses (which assume that a variable can be predicted from a set of other variables and seek the best mathematical relationship between them) were used to study the weight of each cytokine in the normal balance of Th1/Th2 physiological network. This procedure may be helpful in building a model when we have a large number of possible independent variables and are unsure which to include.

Table 1: Cytokine levels were determined in (1) whole blood (wb) and PBMC supernatant (s) in physiological conditions (basic conditions) and after stimulation with PHA (to study T cell network contribution) and LPS (to evaluate the influence of antigen presenting cells) (2) in blood serum. The levels are expressed as mean ± SD and statistical differences between men and women were assessed using the Mann-Whitney U-test or Student's *t*-test as appropriate. Production levels of cytokines did not differ significantly with the exception of IL10 (using the ^{wb} method) which was higher in men than in women when PHA stimulus was used ($P = 0.038$).

(a) Whole blood and PBMC supernatants

Healthy subjects					
Men					
	Basic condition		PHA		LPS
pg/mL	mean^{wb} ± SD	mean^{s} ± SD	mean^{wb} ± SD	mean^{s} ± SD	mean^{wb} ± SD
IL10	5 ± 13	26 ± 40	38 ± 43	94 ± 117	61 ± 96
IFN	149 ± 194	162 ± 146	1752 ± 2344	416 ± 389	1473 ± 2408
IL6	133 ± 376	391 ± 496	236 ± 303	1386 ± 2138	573 ± 715
IL2	256 ± 182	64 ± 78	249 ± 233	290 ± 197	
IL4	15 ± 32	7 ± 10	22 ± 29	22 ± 24	
Women					
	Basic condition		PHA		LPS
pg/mL	mean^{wb} ± SD	mean^{s} ± SD	mean^{wb} ± SD	mean^{s} ± SD	mean^{wb} ± SD
IL10	26 ± 46	155 ± 181	7 ± 9	143 ± 155	73 ± 150
IFN	70 ± 110	173 ± 74	1300 ± 2294	1071 ± 1102	1603 ± 4603
IL6	35 ± 68	243 ± 239	104 ± 156	1921 ± 1654	317 ± 323
IL2	154 ± 140	239 ± 247	222 ± 193	573 ± 438	
IL4	29 ± 48	24 ± 37	77 ± 116	10 ± 5	

(b) Blood serum

*U/mL	Healthy subjects		Colorectal cancer patients		Adenoma patients	
**ng/mL	Men	Women	Men	Women	Men	Women
pg/mL	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
sIl2R*	233 ± 104	258 ± 191	520 ± 306	558 ± 240	237 ± 23	258 ± 52
SIl6R**	49 ± 38	64 ± 45	115 ± 62	139 ± 73	178 ± 47	181 ± 37
IL2	37 ± 29	68 ± 95	15 ± 36	17 ± 60	19 ± 3	86 ± 157
IFN	57 ± 121	67 ± 84	160 ± 193	146 ± 215	124 ± 89	68 ± 50
IL4	11 ± 11	11 ± 9	160 ± 244	141 ± 214	10 ± 4	21 ± 7
IL6	4 ± 11	5 ± 15	178 ± 783	68 ± 110	0.1 ± 0.2	8 ± 10
IL10	3 ± 5	3 ± 9	20 ± 25	52 ± 114	7 ± 5	6 ± 4
TNF	3 ± 9	3 ± 8	13 ± 25	11 ± 24	37 ± 4	131 ± 172
IL1	161 ± 215	171 ± 250	297 ± 300	343 ± 408	66 ± 30	77 ± 27

A forward or backward selection procedure is possible in the latter method. The forward selection begins with no variables (step 0) and adds them one at a time (steps 1, 2, etc.) according to the highest *F*-statistic values. This allows us to control the entry of variables into the model. The backward selection procedure begins with a model containing all the variables (step 0) and eliminates them one at a time (steps 1, 2, etc.) according to the lowest *F*-statistic values. The forward selection is comparable to onset and evolution of the immune response whilst the backward selection procedure is comparable to the physiological return to equilibrium. When we have finished entering and removing variables, the system then estimates the final model using the Graham-Schmidt algorithm to get the most accurate estimates possible and display the model fitting results.

3. Results

3.1. Healthy Subjects: Differences in the Level Network Profiles of Men and Women Are Not, in Theory, Responsible for the Sexually Dimorphic Generation of the Immune Response in Healthy Subjects. Pathological conditions have been found to arise from alterations in the environment Th1/Th2 cytokine network since the relative proportion of each Th1 and/or Th2 cell-type generation, and so the type of immune response, depends on the level of each Th1 and/or Th2-type of cytokine. Our results show that gender-related differences in the immune response in health are not the result of differences between male and female level network profiles, because no significant differences were observed in these profiles, with the exception of IL-10 which was higher in men when PHA stimulus was used (see Table 1).

3.2. Differences in the Relationships in the "Level Network Profiles" However, Could Be Responsible for the Sexual Dimorphism of the Immune Response in Health. In fact, in the evaluation of the Th-cytokine data-driven models of the immune response (Figure 4) it emerged that the level network profile with activated APCs (+LPS, Figure 1) affects the direction of the immune response in both men and women under resting (Basic, Figure 1) and activated (+PHA, Figure 1) conditions.

Hence, APCs (+LPS, Figure 1) regulate the starting type (Basic) and evolution (+PHA) of immunological responses in both men and women, but the effect under resting conditions (Basic, Figure 1), resembling the onset of the immune response (because the cells are in the resting state), appears to be exerted by IFNγ production in men, and by IL-6 in women, whilst in activated conditions (PHA, Figure 1), resembling the evolution of the immune responses (because the cells are in the activation state), by IL-6 production again in women but by IFNγ and IL-6 in men.

The network profile of the production levels of Th1 and Th2 cytokines (*level network profile*) in resting (Basic, in the sense of unstimulated) conditions does not appear to have a specific role in T cell differentiation in men since no significant relationships were found in whole blood culture supernatants without stimulus (Figure 1). In women, this regulation would appear to be exerted through a Th1 and Th2 linked production of IL-2 (Th1) and IL-4 (Th2), IFNγ (Th1) and IL-6 (Th2) cytokines (Figure 1). Interestingly, this IL-2 and IL-4 Th1/Th2 interregulation in women seems to have both an early and late role in the control of the Th1 and Th2 cell network since the relationships between their levels are significant in both the 24-hour and 72-hour culture supernatant cytokine assays. The interregulation between IL-6 and IFNγ levels is only significant in the 72-hour assay (cytokine assay of the whole blood supernatant after 72 h of cell culture).

Additionally, in Figure 1 it would appear that the early differentiation of activated T cells (+PHA after 24 h of culture) is influenced by the positive linked production of IL-6 and IL-4, IFNγ and IL10 cytokines in men, and the negative linked production of IL-6 and IL-10 cytokines in women. Likewise the late Th1 or Th2 differentiation of activated T cells (+PHA after 72 h of culture) seems to be influenced by the positive linked production of IFNγ and IL-4 in men, while by IL-6 and IFNγ in women.

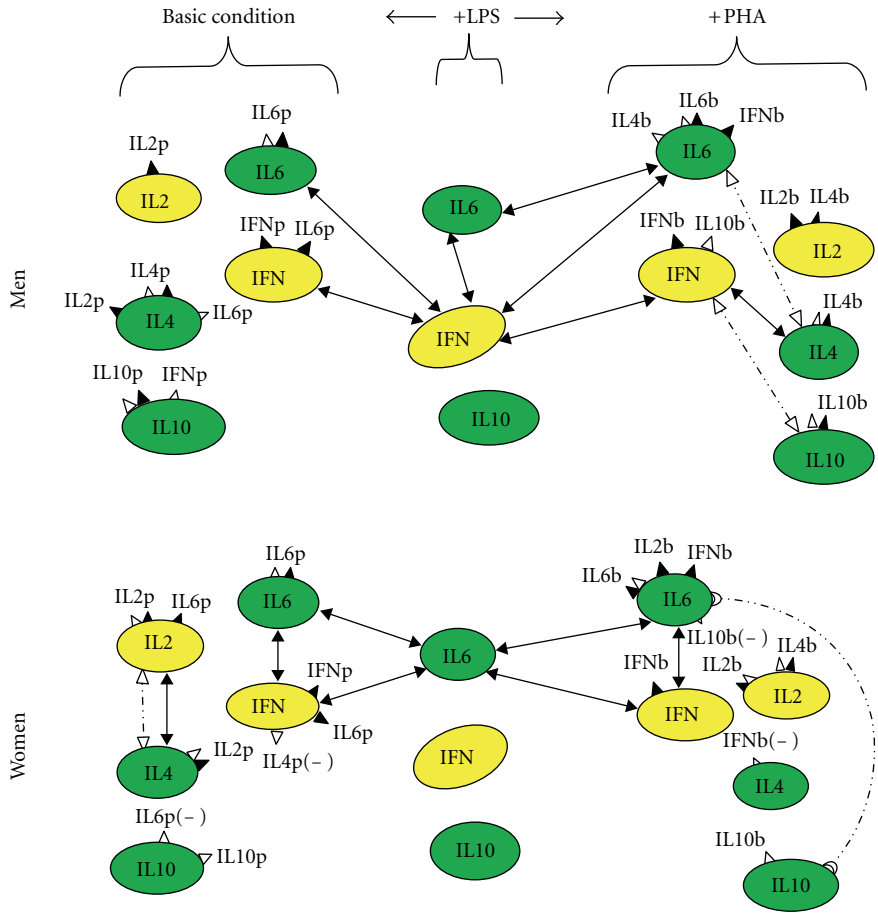
3.3. Differences between Men and Women in the Relationships of Serum "Level Network Profiles" Could Represent Possible Gender Biomarkers for Sexually Dimorphic Generation of Immune Responses in Health and Disease States. Gender-specific and gender-common significant Th1/Th2 network relationships were found in serum in men and women (Figure 2). A gender-specific biomarker in resting conditions (Figure 4) may be the positive relationship between IL-2 and IL-6 cytokines which was significant in men but not women (Figures 2 and 5: "T cell"). Gender-specific biomarkers in activation conditions (Figure 4) may be the positive relationships between sIL-2R and IFNγ, sIL6-R, and sIL-2R (Figures 2 and 5: "T cell") which again were significant in men, but not in women. No women gender-specific relationships were found in resting conditions but positive relationships between sIL-2R and IL-4, sIL-6R and IFNγ were identified in activation conditions (Figure 5) that could be used as biomarkers. The positive relationship between sIL-6R and IL-4 and the negative one between sIL6R and IL6 may represent common activation biomarkers for both men and women.

The negative relationships between IL-1β and IL-2, TNFα and IL-1β, TNFα and IFNγ, and TNFα and IL-4 and the positive one between TNFα and IL-6 (Figures 2 and 5: "APC"), are possible male gender specific biomarkers for APC T cell differentiation in men in resting conditions (Figure 5). There were no female APC gender-specific biomarkers in resting conditions, whilst a common biomarker in resting conditions appears to be the positive relationship between IL-1β and IL-4 (Figures 2 and 5). Finally, the positive relationships between IL-1β and sIL6R in men and IL-1β and sIL-2R in women (Figure 2) are possible gender-specific biomarkers for APC T cell differentiation in activated conditions, whereas the negative relationship between TNFα and sIL-6R a common APC biomarker (Figure 5).

Additionally age in men was related to IL-1β (c.coef. = 0.45, $P = 0.010$) and in women to IFNγ (c.coef. = 0.71, $^*P < 0.0001$), sIL-6R (c.coef. = 0.51, $P = 0.004$) and IL-10 (c.coef. = -0.35, $P = 0.047$).

3.4. Colorectal Cancer and Adenoma Patients: Differences between Men and Women in "Level Network Profiles" Are Not Responsible for the Sexually Dimorphic Generation of Immune Responses in Disease but as Discussed in the Last Section, Differing Responses May Arise from Differences in the Relationships within "Level Network Profiles". In order to confirm our results on healthy subjects, independent cohorts of colorectal cancer and adenoma patients were also assessed using the same Th-cytokine data-driven computational models. No significant differences were found between men and women in serum "level network profiles" in both colorectal cancer and adenoma patients, confirming our results concerning healthy subjects (Table 1). In addition, significant alterations in the IFNγ and IL-6 gender-specific pathways and IL-10 gender-common pathways were found in colorectal cancer patients (Figure 2). Further confirmation also came from the finding that in adenoma patients, gender-specific pathways IFNγ and IL-6 still partially regulate immune response homeostasis in men and women and in neither sex was a significant relationship observed between IL-10 and the other Th1/Th2 network components (Figure 3).

3.5. Colorectal Cancer Patients: Alterations in the Relationships of IFNγ and IL-6 Gender-Specific Pathways and of IL-10 Gender-Common Pathways Are Biomarkers for the Loss of Immune Response Homeostasis and Disease Progression in Both Men and Women, but through Gender-Specific Mechanisms. No significant relationships with IFNγ were observed in the male group or with IL-6 in the female group indicating alterations in the gender-specific Th-cytokine pathways (Figure 2, healthy subjects and colorectal cancer patients). Significant relationships between IL-10 and other Th1/Th2 network components were observed in both men and women



▲ ⟷ Positive relationships (72 h assay)

△ ◁···▷ Positive relationships (24 h assay)

☽···☾ Negative relationships (24 h assay)

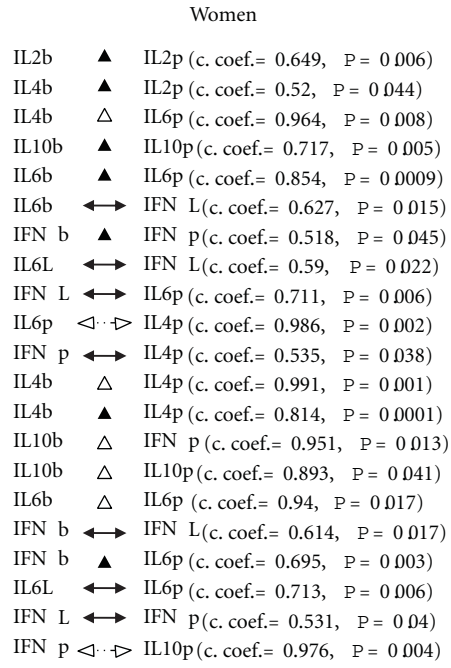
(a)

Men

IL2b	▲	IL2p (c. coef. = 0.705, P = 0.022)
IL2b	⟷	IL4b (c. coef. = 0.661, P = 0.047)
IL2b	▲	IL6p (c. coef. = 0.697, P = 0.025)
IL4b	▲	IL2p (c. coef. = 0.772, P = 0.021)
IL10b	△	IL6p (c. coef. = −0.933, P = 0.02)
IL6b	▲	IL6p (c. coef. = 0.878, P = 0.0008)
IL6b	⟷	IL6L (c. coef. = 0.738, P = 0.0027)
IFNb	▲	IFNp (c. coef. = 0.668, P = 0.035)
IFNb	⟷	IL6L (c. coef. = 0.812, P = 0.015)
IL6L	⟷	IL6p (c. coef. = 0.779, P = 0.019)
IL6p	⟷	IFNp (c. coef. = 0.693, P = 0.037)
IL2b	△	IL2p (c. coef. = 0.92, P = 0.0027)
IL2b	◁···▷	IL4b (c. coef. = 0.936, P = 0.019)
IL4b	△	IL2p (c. coef. = 0.958, P = 0.01)
IL10b	△	IL10p (c .coef. = 0.991, P = 0.001)
IL6b	⟷	IFNb (c. coef. = 0.901, P = 0.0004)
IL6b	△	IL6p (c. coef. = 0.917, P = 0.028)
IFNb	△	IL4p (c. coef. = −0.901, P = 0.036)
IFNb	▲	IL6p (c. coef. = 0.796, P = 0.006)
IL6L	⟷	IFNp (c. coef. = 0.758, P = 0.023)
IL6p	☽···☾	IL10p (c. coef. = −0.953, P = 0.012)

(b)

Figure 1: Continued.



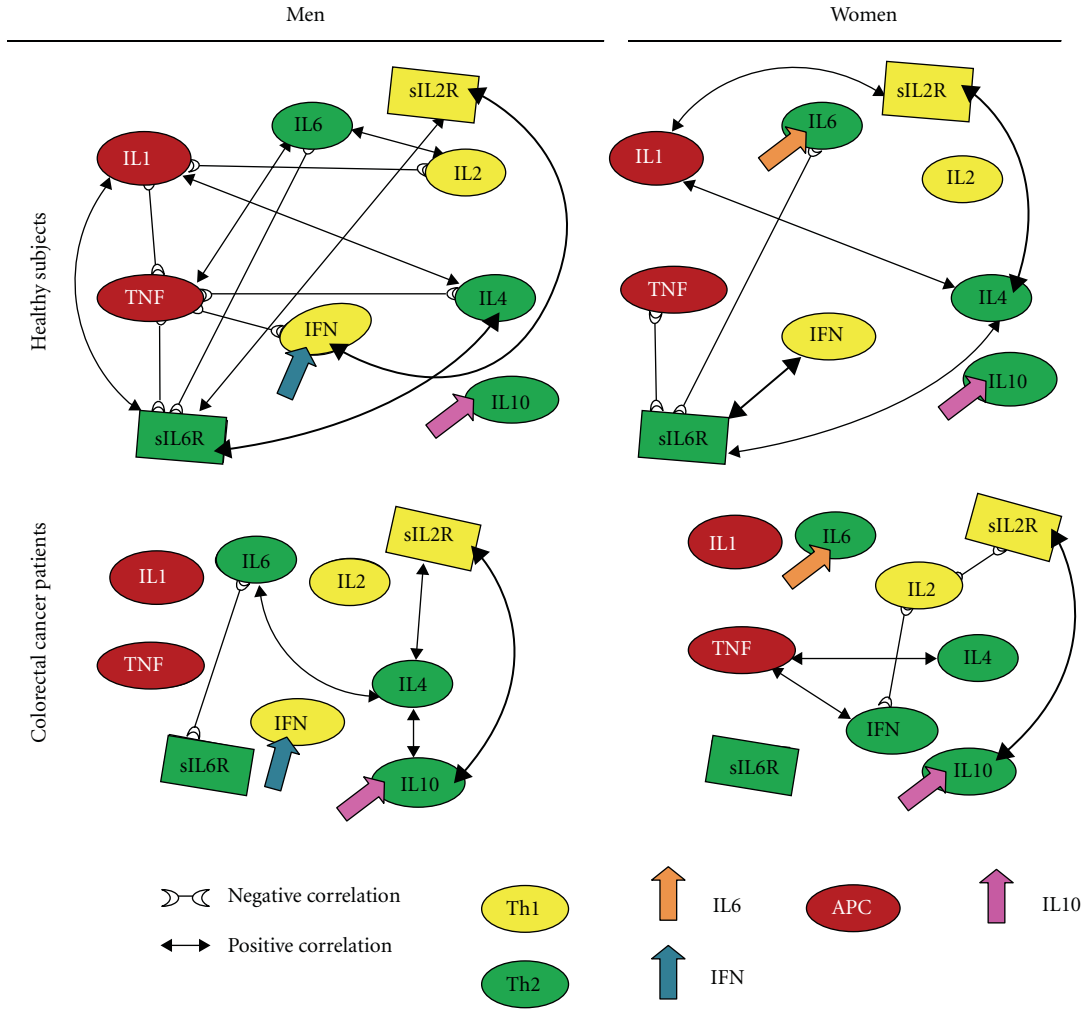
(c)

Figure 1: Relationships using the whole blood assay method (72h assay, black): (black triangle) Positive relationships. Relationships using PBMC cells separated by Ficoll/Hypaque gradient procedures (24 h assay, white): (white triangle) positive and (crescent shape) negative relationships. Basic Condition: resting state; +LPS: APC activated condition; +PHA: T cell activated condition. Basic condition (b), LPS (L), PHA (p). In healthy subjects, differences in the relationships in the "level network profiles" could be responsible for the sexual dimorphism of the immune response in health. Gender-specific relationships in "level network profiles" affect the direction (Th1 or Th2) of the immune response under resting (Basic) and activated (+PHA) conditions. APCs (+LPS) regulate the starting type and evolution of immunological responses in both men and women: the starting type (+LPS → Basic,) appears to be regulated by IFNγ production in men, and by IL6 in women; the evolution (+LPS → +PHA) by continuing IL6 production in women and by IFNγ in men. No significant relationships were found in whole blood culture supernatants without stimulus (basic conditions) in men. In women, this regulation would appear to be exerted by the linked production of IL2, IL4 and IFNγ, IL6 cytokines (relationships in basic conditions). The IL2 and IL4 interregulation in women seems to have both an early and late role since the correlation between their levels are significant in both the 24h and 72h culture supernatant cytokine assays; the interregulation between IL6 and IFNγ levels only has a late function because it is only significant in the 72h assay. The earlier evolution of activated T cells (+PHA 24h culture) seems to be influenced by the linked production of IL6 and IL4, IFNγ and IL10 cytokines in men; while by IL6 and IL10 cytokines in women. The late evolution of activated T cells (+PHA 72h whole blood of culture) on the other hand seems to be influenced by the linked production of IFNγ and IL4 in men, while by IFNγ and IL6 in women.

groups, but through different Th1/Th2 pathways, indicating alterations in gender common Th-cytokine pathways. However, sexual dimorphism in cytokine relationships included (Figures 2 and 5) the following: (1) a positive relationship between serum levels of IL-6 and IL-4 in the male group, which in its turn was positively correlated to IL-10 levels; (2) a negative relationship between IL-2 and IFNγ in the female group; (3) positive relationships between serum levels of sIL-2R activation biomarker and IL-10 in men, in addition to the relationship between sIL-2R and IL-4; and (4) a negative relationship between sIL-2R and IL-2, and a positive one between sIL-2R and IL-10 in the female group. Finally, positive relationships between TNFα, the APC biomarker, and IFNγ and IL-4 were again found in the female group (Figure 2). However, no relationships between APC biomarkers in activation conditions (Figure 4) were found in either group (Figures 2 and 5).

In fact, we found that patient disease progression (stage correlation) was related to an increase of IL-10 (men: c.coef. = 0.61, $P = 0.002$; women: c.coef. = 0.81, $P = 0.002$) and sIL-2R (men: c.coef.=0.39, $P = 0.048$; women: c.coef.=0.70, $P = 0.009$)) in both sexes, but in men disease progression is also related to an increase of IL-4 (c.coef. = 0.49, $P = 0.014$) and IL-6 (c.coef. = 0.42, $P = 0.034$), while in women to a decrease in IL-2 (c.coef. = −0.58, $P = 0.031$). Moreover in women age is linked to a decrease of TNFα (c.coef. = −0.67, $P = 0.012$) and sIL-2R (c.coef.= −0.57, $P = 0.033$).

3.6. Adenoma Patients: The Relationships Described Represent Gender-Specific Biomarkers for the Passage from Health to Adenoma and Colorectal Cancer Disease. Gender-specific IFNγ (men) and IL-6 (women) pathways still partially regulate Th1 and Th2 cell network homeostasis in adenoma patients, in contrast to colorectal patients (Figure 3). Under immune resting conditions (Figure 4), the significant positive relationship between IL-6 and IL-4 indicates that IL-6 pathways were still operating within the Th1/Th2 network in the group of female patients (Figures 3 and 5). No significant



Men		
IL1	↔	sIL6R (c. coef. = 0.59, P = 0.0008)
IL1	⟩—⟨	TNF (c. coef. = −0.48, P = 0.007)
IL1	⟩—⟨	IL2 (c. coef. = −0.45, P = 0.01)
IL1	↔	IL4 (c. coef. = 0.69, P = 0.0001)
TNF	⟩—⟨	sIL6R (c. coef. = −0.52, P = 0.003)
TNF	⟩—⟨	IL4 (c. coef. = −0.52, P = 0.003)
TNF	⟩—⟨	IFN (c. coef. = −0.42, P = 0.016)
TNF	↔	IL6 (c. coef. = 0.37, P = 0.036)
sIL6R	⟩—⟨	IL6 (c. coef. = −0.37, P = 0.038)
sIL6R	↔	sIL2R (c. coef. = 0.45, P = 0.011)
sIL6R	↔	IL4 (c. coef. = 0.45, P = 0.011)
sIL2R	↔	IFN (c. coef. = 0.37, P = 0.037)
IL6	↔	IL2 (c. coef. = 0.38, P = 0.03)

Women		
IL1	↔	IL4 (c. coef. = 0.53, P = 0.003)
IL1	↔	sIL2R (c. coef. = 0.55, P = 0.002)
sIL2R	↔	IL4 (c. coef. = 0.50, P = 0.005)
IL4	↔	sIL6R (c. coef. = 0.39, P = 0.026)
sIL6R	↔	IFN (c. coef. = 0.67, *P < 0.0001)
TNF	⟩—⟨	sIL6R (c. coef. = −0.37, P = 0.03)
sIL6R	⟩—⟨	IL6 (c. coef. = −0.63, P = 0.0003)

(a)

Figure 2: Continued.

Men			Women		
sIL6R	⟩—⟨	IL6 (c. coef. = −0.54, P = 0.007)	TNF	⟷	IFN (c. coef. = 0.65, P = 0.015)
IL6	⟷	IL4 (c. coef. = 0.40, P = 0.044)	TNF	⟷	IL4 (c. coef. = 0.59, P = 0.026)
sIL2R	⟷	IL4 (c. coef. = 0.49, P = 0.014)	sIL2R	⟷	IL10 (c. coef. = 0.62, P = 0.021)
IL4	⟷	IL10 (c. coef. = 0.54, P = 0.006)	IL2	⟩—⟨	IFN (c. coef. = −0.56, P = 0.035)
sIL2R	⟷	IL10 (c. coef. = 0.50, P = 0.013)	IL2	⟩—⟨	sIL2R (c. coef. = −0.75, P = 0.005)

(b)

Figure 2: Differences between men and women in the relationships of serum "level network profiles" could represent possible gender biomarkers for sexually dimorphic generation of immune responses in health and disease states. In healthy subjects (a), significant gender-specific and gender-common Th1/Th2 network relationships were found in serum which could be used as biomarkers to identify the direction of T cell differentiation. However, in neither sex did the IL10 cytokine interact with other network components. In colorectal cancer patients (b) no significant relationships with IFNγ in the male group were observed or with IL6 in the female group, indicating alterations in the gender-specific Th-cytokine pathways; significant relationships between IL10 and other Th1/Th2 network components were observed in both men and women groups indicating alterations in the gender-common pathways but through different Th1/Th2 pathways.

Th1 polarization biomarkers were found in either sex under activation conditions; Th2 polarization biomarkers, on the other hand, were linked to an increase of sIL-2R and IL-4 in men, and sIL-2R and IL-6 plus sIL-2R and IL-4 in women (Figures 3 and 5).

Under immune resting conditions (Figure 4) the significant negative relationship between TNFα and IFNγ, and the positive one between IL-1β and IFNγ levels, in addition to TNFα and IL-2, indicate that APC IFNγ pathways were still operating within the Th1/Th2 network model under basic conditions in men (Figures 3 and 5). In women, the influence of APCs under basic conditions emerges from positive relationship between TNFα and IL-2 (Figures 3 and 5). APCs do not seem to influence Th1 and Th2 cell network homeostasis under activation conditions (Figure 4). In fact, no statistically significant relationships were found between soluble molecules (sIL-2R and sIL-6R) and TNFα or IL-1β in either group (Figures 3 and 5).

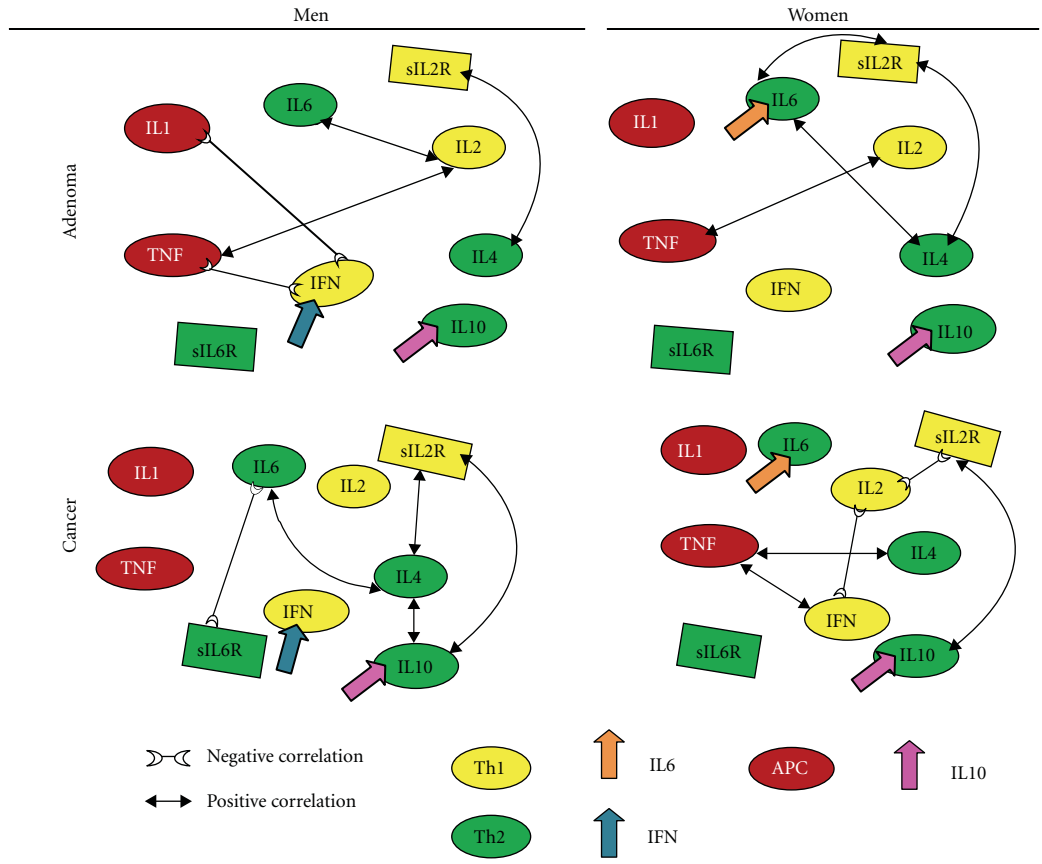
Even if the results of the adenoma study should be handled with prudence considering the number of patients, IFNγ and IL-6 pathways partially regulate Th1 and Th2 cell network homeostasis (IFNγ in men and IL-6 in women, resp.). However, in neither sex was a significant relationship observed between IL-10 and other Th1/Th2 network component which should be short-lived in both sexes. Therefore, IL-10 environment persistence is a biomarker for the loss of the regulatory mechanisms responsible for restoring the initial Th1/Th2 physiological equilibrium [47] in men and women.

4. Significant Independent Factors for Predicting Alterations in Immune Response Homeostasis Regulation of Common and Gender-Specific Th-Pathways

The stepwise multiple regression analysis, using the forward procedure, allowed us to identify the greatest weighting parameters on IFNγ and IL-6 gender-specific pathways and IL-10 gender-common pathways. The results also indicate that the serum level of IFNγ ($P = 0.0001$) in men could be a significant independent factor for predicting a possible alteration in IL-10 regulation of the balance between Th1 and Th2 cell types (Figure 6). The independent factors sIL-2R ($P = 0.0004$) and IL-10 ($P = 0.0001$) are, on the other hand, important for predicting an alteration in the normal regulation that IFNγ exerts over the balance between Th1 and Th2 cell types. In women (Figure 6) sIL-2R ($P = 0.041$) and IL-4 ($P = 0.003$) may prove useful as significant independent factors to predict alterations in the normal regulation that IL-10 exerts over the balance between Th1 and Th2 cell types; likewise sIL-6R ($P < 0.0001$) and IFNγ ($P < 0.0001$) may prove useful as significant independent factors to predict alterations in the normal regulation that IL-6 exerts over the balance between Th1 and Th2 cell types. The results of multiple regression analysis show that age could also be a significant independent factor for IFNγ ($P = 0.01$) and IL-10 ($P = 0.03$) in men; whilst in women age appears to be significant for sIL-6R ($P = 0.002$) and IFNγ ($P = 0.04$).

5. Discussion

We put forward the hypothesis that gender-dependent immune responses in health and disease states and differing reactions to disease and therapy could be due to gender-specific Th1/Th2 production pathways. The identification of these gender-specific pathways and the correlated targets/biomarkers could lead to more specifically tailored treatment and better therapeutic success rates. In order to test this hypothesis, we decided to study and evaluate the possibility of using Th1 and Th2 cytokines as biomarkers in immune response models, as they are responsible for propelling the immune response in a given Th1 or Th2 direction: the "level network profile" (the network profile of the production levels of Th1 and Th2 cytokines) by APCs is indicative of the direction of T cell differentiation during the immune response, and the balance between their levels and between their relationships indicates a normal Th1 and Th2 cell differentiation and so a productive immune response; a lack of balance indicates pathology. We developed Th-cytokine



(a)

Men

IL6 ⟷ IL2 (c. coef. = 0.9, P = 0.019)
IL1 ⟜⟝ IFN (c. coef. = −0.9, P = 0.005)
sIL2R ⟜⟝ IL4 (c. coef. = −0.9, P = 0.046)
TNF ⟷ IL2 (c. coef. = 0.9, P = 0.019)
TNF ⟜⟝ IFN (c. coef. = −0.9, P = 0.005)

Women

sIL2R ⟷ IL4 (c. coef. = 0.9, P = 0.0004)
sIL2R ⟷ IL6 (c. coef. = 0.9, P = 0.0004)
IL6 ⟷ IL4 (c. coef. = 0.9, P = 0.002)
TNF ⟷ IL2 (c. coef. = 0.9, P = 0.028)

(b)

Men

sIL6R ⟜⟝ IL6 (c. coef. = −0.54, P = 0.007)
IL6 ⟷ IL4 (c. coef. = 0.40, P = 0.044)
sIL2R ⟷ IL4 (c. coef. = 0.49, P = 0.014)
IL4 ⟷ IL10 (c. coef. = 0.54, P = 0.006)
sIL2R ⟷ IL10 (c. coef. = 0.50, P = 0.013)

Women

TNF ⟷ IFN (c. coef. = 0.65, P = 0.015)
TNF ⟷ IL4 (c. coef. = 0.59, P = 0.026)
sIL2R ⟷ IL10 (c. coef. = 0.62, P = 0.021)
IL2 ⟜⟝ IFN (c. coef. = −0.56, P = 0.035)
IL2 ⟜⟝ sIL2R (c. coef. = −0.75, P = 0.005)

Figure 3: In adenoma patients (a), gender-specific pathways partially regulate Th1 and Th2 cell network homeostasis. In neither sex was a significant relationship observed between IL10 and other Th1/Th2 network components. IFNγ and IL6 pathways (in men and women, resp.) still regulate, albeit partially, the sex-specific Th1 and Th2 cell network homeostasis (and so the immune response) in adenoma patients; in neither sex was a significant relationship observed between IL10 and other Th1 and Th2 network cytokines. No significant relationships for IFNγ or IL6 (in men and women, resp.) were observed in colorectal cancer patients (b), indicating alterations in the gender-specific regulatory pathways responsible for Th1/Th2 physiological homeostasis. The persistence of IL10 within the environmental network is a significant biomarker for the loss of Th1 and Th2 cell network homeostasis and disease progression in both men and women, mediated however through different sex-related Th1/Th2 pathways. In normal immune response the influence of IL10 on Th polarization is short-lived in both sexes.

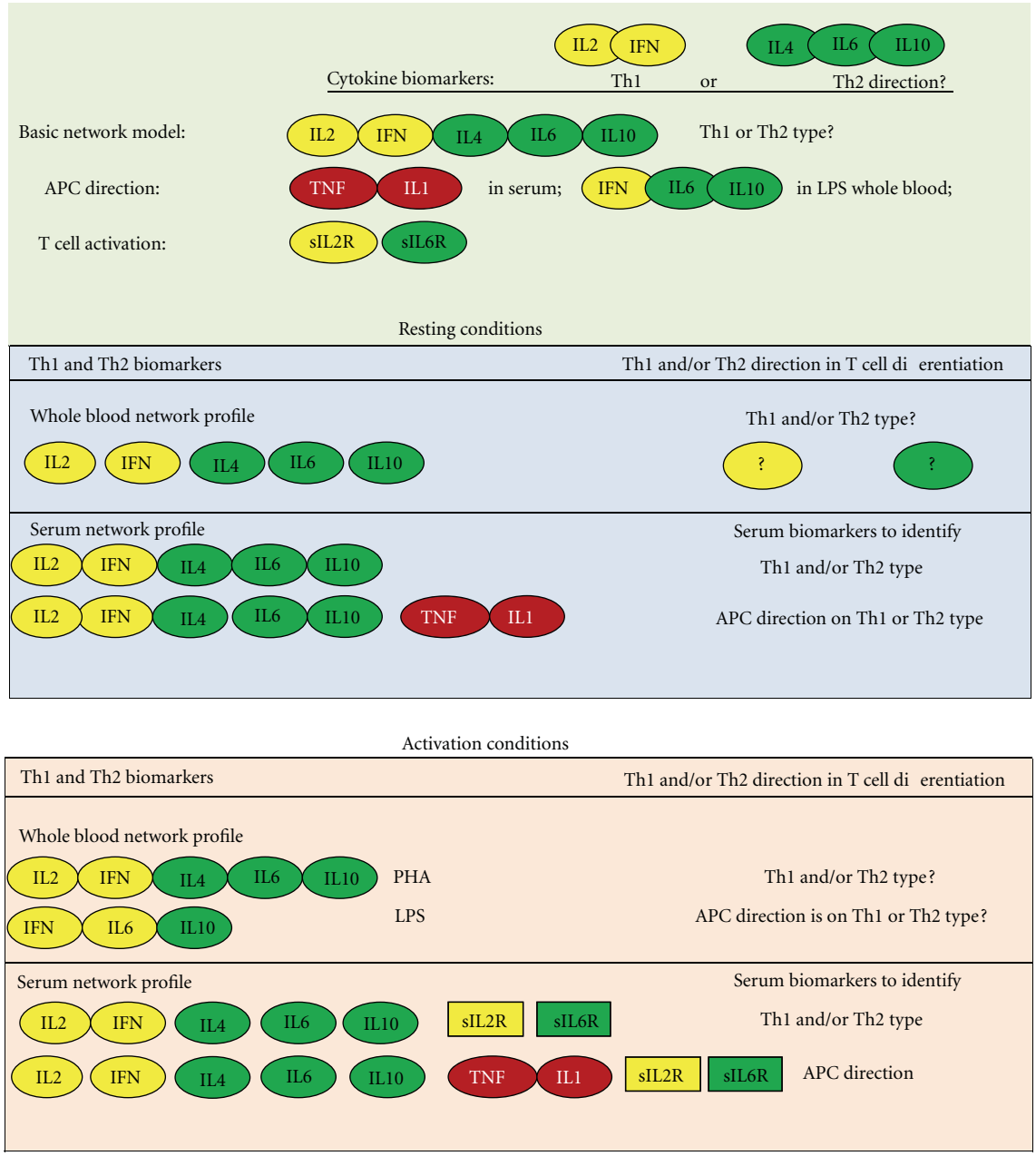


Figure 4: Th-cytokine models of the immune response in resting and activation conditions: whole blood levels of specific Th1 and Th2 cytokines were used as biomarkers in Th-cytokine data-driven computational models of the immune response to determine the direction of T cell differentiation (Th1 or Th2). The cytokines used in our Th-cytokine data-driven computational models of the immune response were: interleukin (IL)-2, interferon (IFN)γ, IL-4, IL-6, and IL-10, to make up our basic network model, to establish if the direction in T cell differentiation was Th1 and/or Th2 type; tumor necrosis factor (TNF)α and IL-1β as serum biomarkers and IFNγ, IL-6, and IL-10 as LPS whole blood biomarkers, to determine if APC direction on T cell differentiation was of Th1 or Th2 type; and soluble (s) IL-2 receptor (R) and sIL-6R to estimate cell activation. APC: antigen presenting cells; PHA: to study T cell network contribution; LPS: to evaluate the influence of antigen presenting cells.

data-driven models of the immune response (Figure 4) and evaluated peripheral blood samples of healthy subjects. To back up our results, independent cohorts of colorectal cancer and adenoma patients were also evaluated. Our hypothesis was confirmed since our results not only indicate that gender-specific treatment should improve therapeutic success rates but also highlight the importance of peripheral blood Th1/Th2 network pathways as physiological targets/biomarkers in clinical investigations and translational pharmacology research.

The results of this study indicate, for the first time, that physiological gender-specific Th1/Th2 pathways regulate the homeostasis of the Th1/Th2 cell network and hence the immune response. These gender pathways are therefore

	Healthy subjects		Adenoma patients		Colorectal cancer patients	
T cell:	Men	Women	Men	Women	Men	Women
RC	IL2(+)IL6	ns	L2(+)IL6	IL4(+)IL6	IL4(+)IL6 IL4(+)IL10	IL2(−)IFN
AC	sIL2R(+)IFN	sIL2R(+)IL4	sIL2R(+)IL4	sIL2R(+)IL4	sIL2R(+)IL4 sIL2R(+)IL10	IL2R(−)IL2 sIL2R(+)IL10
	sIL6R(+)IL4	IL6R(+)IFN	ns	ns	ns	ns
	sIL6R(−)IL6	sIL6R(−)IL6	ns	ns	sIL6R(−)IL6	ns
	sIL6R(+)sIL2R	sIL6R(+)IL4	ns	ns	ns	ns
APC	Men	Women	Men	Women	Men	Women
RC	TNF (+)IL6	IL1 (+)IL4	TNF (+)IL2	TNF (+)IL2	ns	TNF (+)IL4
	TNF (−)IL4	ns	ns	ns	ns	ns
	TNF (−)IFN	ns	TNF (−)IFN	ns	ns	ns
	TNF (−)IL1	ns	ns	ns	ns	ns
	IL1 (+)IL2	ns	IL1 (+)IFN	ns	ns	ns
AC	TNF (−)sIL6R	TNF (−)sIL6R	ns	ns	ns	ns
	IL1 (+)sIL6R	IL1 (+)sIL6R	ns	ns	ns	ns

(+) Positive relationship
(−) Negative relationship
RC Resting
AC Activation conditions
(ns) Not significant
Th1 IL2, IFN
Th2 IL4, IL6, IL10
APC TNF , IL1

Figure 5: Serum Th1 and Th2 network relationships. Significant gender common and gender-specific Th1/Th2 network relationships were found in serum which could be used as biomarkers to indicate the direction of T cell differentiation in the immune response. Positive relationship (+); negative relationship (−); resting (RC) and activation (AC) conditions; not significant (ns); Th1: IL2, IFNγ; Th2: IL4, IL6, IL10; APC: TNFα, IL1β.

probably responsible for gender-dependent reactions to disease and therapy as a consequence of their specific, regulatory roles in Th cell polarization during the development of the immune response and in restoring physiological homeostasis.

The new points that emerge from our study can be summed up as follows: (1) IFNγ and IL-6 production pathways are respectively male and female gender-specific health pathways for immune response homeostasis (Figure 1) and consequently targets and/or biomarkers for the passage from health to adenoma and colorectal cancer (Figures 2 and 3); (2) the IL-10 pathway is a common-gender pathway involved in restoring immune system resting homeostasis (Figure 1), but only if controlled by the respectively gender specific pathways; otherwise it is a cancer progression target/biomarker (Figures 2 and 3); (3) the gender specific differences in serum "level network profiles" represent significant biomarkers that could be used to develop more specific approaches (Figures 5 and 6).

In more detail, our results showed that gender specific IFNγ and IL-6 pathways respectively regulate male and female immune response homeostasis, however in neither sex were significant relationships observed between IL-10 and other Th1/Th2 network components, apart from that between IL-10 and IFNγ in the male group and IL-10 and IL-6 in the female group precociously in the cellular network (24 h) (Figure 1). In order to maintain a normal balance between Th1 and Th2 cells, the effect of IL-10 on Th polarization must be, therefore, short-lived and in the linked positive production of IFNγ and IL-10 cytokines in men and the negative production of IL-6 and IL-10 in women, IFNγ and IL-6 pathways could be considered gender specific pathways for the regulation of immune system homeostasis (Figure 1). APC regulation in Th1 and Th2 cell network homeostasis also appeared to be exerted through IFNγ production in men, and IL-6 production in women in both resting and activated conditions (Figure 1, correlation between the results of LPS and basic and PHA stimulus, resp.). So it would appear that IFN and IL-6 Th-cytokine pathways are gender specific targets and biomarkers for the onset and development of the immune responses, whilst IL-10 Th-cytokine pathways operate in the same way in both sexes, regulating the recovery of homeostatic equilibrium within the Th1 and Th2 cell network at the end of the immune response.

The above results in healthy subjects were confirmed by the results in the adenoma and colorectal cancer disease groups. In fact, within our colorectal cancer patient group we noted alterations in the IFNγpathways in men and IL-6 in women and persistence of IL-10 under both resting and activated conditions (Figure 2). In the adenoma group, on the other hand, IFNγ and IL-6 pathways still partially regulated

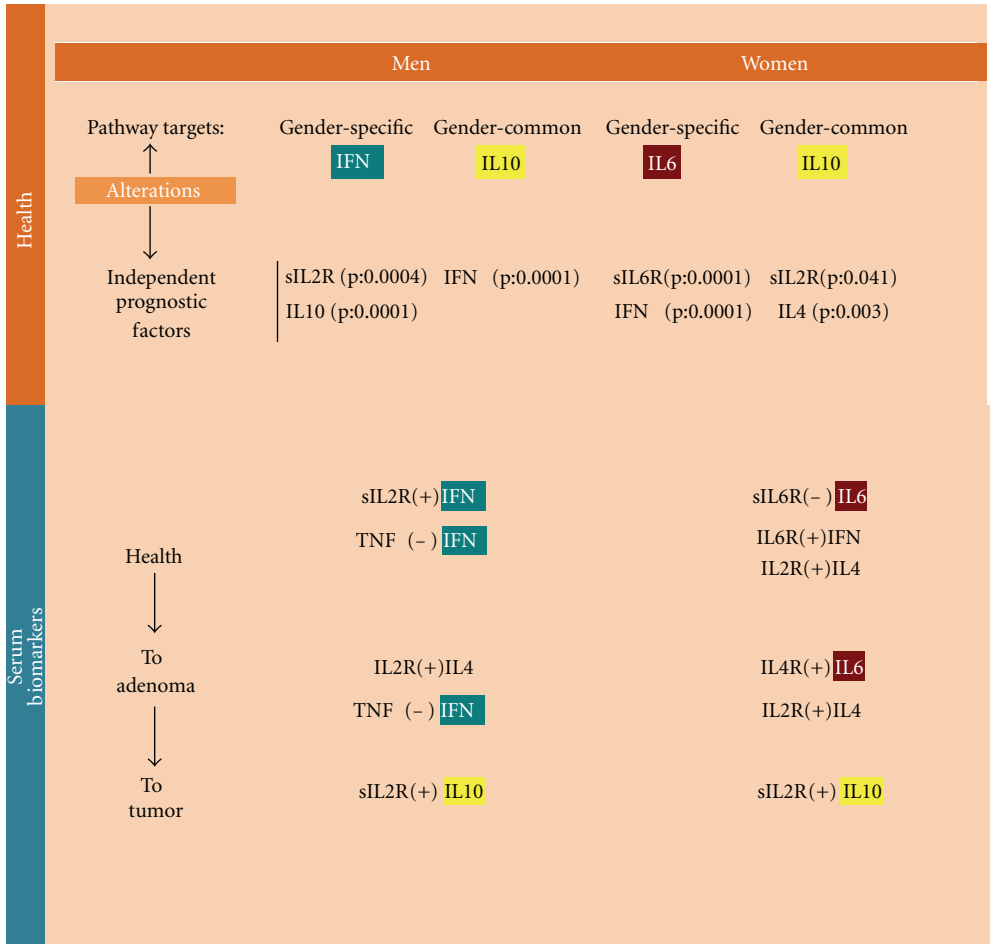


Figure 6: The results of the stepwise multiple regression analysis (using the forward procedure) indicate that the serum level of IFNγ in men could be a significant independent factor for predicting a possible alteration in IL10 regulation of the balance between Th1 and Th2 cell types. The independent factors sIL2R and IL10 are important for predicting an alteration in the normal regulation that IFNγ exerts over the balance between Th1 and Th2 cell types. In women sIL2R and IL4 may prove useful as significant independent factors to predict alterations in the normal regulation that IL10 exerts over the balance between Th1 and Th2 cell types; likewise sIL6R and IFNγ may prove useful as significant independent factors to predict alterations in the normal regulation that IL6 exerts over the balance between Th1 and Th2 cell types.

gender specific Th1 and Th2 cell network homeostasis but in neither sex was a significant relationship observed between IL-10 and other Th1/Th2 network components (Figure 3).

Our results indicate that in the normal mucosa through adenoma to tumor progression, the host immune response proceeds from a physiological condition, where gender-specific Th1/Th2 pathways regulate the homeostasis of the Th1/Th2 cell network, to a type with partial or absent gender-specific Th1/Th2 pathways regulation and immunological suppressive characteristics (adenoma and cancer patients). Moreover, in the adenoma patients there was no IL-10 involvement, while this parameter was implicated in the cancer patients' immune responses, suggesting that IL-10 may be prognostic for the passage from adenoma to cancer as a dual biomarker together with sIL-2R (Figures 1, 2, 3, and 6).

In fact in healthy subjects, the sIL-2R in men and sIL-6R in women were principally related to IFNγ (Figures 2 and 6), which plays an important role in the development of Th1 cells; in adenoma the TNFα in men and IL-4 in women were, respectively, related to IFNγ and IL-6 (Figures 3 and 6). So in the adenoma patients, gender-specific Th1/Th2 pathways were involved in the Th1/Th2 network, while IL-10 (immunologically suppressive) was excluded; in the patient group there was an inverted situation.

Since the stepwise nature of colorectal cancer has been well defined and colon adenoma has been identified as a precursor of colorectal cancer, colon adenoma is a particularly meaningful intermediate outcome for studying factors related to colorectal cancer. Therefore, the differences observed between colon adenoma and colorectal cancer patients confirm that the IFNy production pathway for immune response homeostasis is specific to men, while the IL-6 production pathway for immune response homeostasis is specific to women. The IL-10, pathway for restoring immune system resting homeostasis was common to both but was controlled by the respective gender-specific pathways. In this way our hypothesis is confirmed: gender-dependent immune responses in health and disease states and differing reactions

to disease and therapy could be due to gender-specific Th1/Th2 production pathways. These gender-specific pathways and the correlated targets/biomarkers (Figure 6) could lead to more specifically tailored treatment and better therapeutic success rates.

In fact, the observations made in this study may be useful for gender-specific therapeutic strategies. In men (Figures 2 and 6) changes in the level of IFNy, IL-10, and sIL-2R within the physiological normal ranges and high levels of the dual target sIL-2R/IFNy are biomarkers of immunological homeostasis and therapeutic success. Instead, significantly high levels of the dual target sIL-2R/IL-10 are biomarker of immune deficiency and treatment failure. Likewise in women (Figures 2 and 6) changes in the levels of sIL-6R, IFNy, sIL-2R, IL-4, and IL-10 within the physiological normal ranges and high levels of the dual targets sIL-6R/IFNy and sIL-2R/IL-4 are biomarkers of immunological homeostasis and therapeutic success. Instead, significantly high levels of the dual target sIL-2R and IL-10 are biomarker of immune deficiency and treatment failure.

The mechanisms responsible for gender-specific disease susceptibility have yet to be clarified. However our data suggest that the answer may lie in the differing capacity of cells to defend themselves against oxidative stress [48]. The cells of men and women differ greatly in terms of reactive oxygen species production and oxidative stress susceptibility [48–50] and this appears to be a promising new field of investigation. In all cell types it has been found, for example, that oxygen metabolism can lead to the production of reactive oxygen species (ROS) such as radicals. All cell types, including lymphocytes and other immune system cells, present a complex range of antioxidant compounds and enzymes, such as glutathione (GSH) and thioredoxin reductasi (TRX) [51, 52] to neutralize ROS and to preserve the cell oxidative balance. Gender-associated redox features of cells have also been described [49, 50]. The activities of ROS, for example, appear to be regulated differently in males and females and can be directly influenced by sex hormones [49, 50].

In vivo studies have further demonstrated the incapacity in males, but not in females, of maintaining intracellular reduced redox conditions, essential for normal cellular functions [48]; this explains, at least in part, the differences between the two sexes in the maintenance of the immune system homeostasis which we observed. In fact, if as has been proposed, IFNγ is a direct stimulator of PBMC thioredoxin and thioredoxin reductase (RTrx) system gene expression in human T cells [53, 54] and there is a positive feed-back circuit involving IFN-γ and Trx/RTrx gene expression in the regulation of intracellular reduced oxitative condition which is essential for Th1 immune response, then we can assume that the immunological response through the IFNγ pathway in men reduces the intracellular oxidative levels to preserve the cell oxidative balance control. In fact, male cells, as we mentioned, are incapable of maintaining an intracellular reduced oxidative condition and this would explain their greater susceptibility to diseases in which the immunological defense is prevalently Th1 type, such as tumors [47]. Similarly if we consider that the key function of IL-6 is the homeostasis within the Th cell differentiation in Treg or Th17 cells [33, 34], it is clear why women are more susceptible to diseases characterized by a lack of regulatory cell functionality such as autoimmune diseases [33, 55].

Conflict of Interests

The authors declare that there are no competing financial interests.

Acknowledgments

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Research Article

The Therapeutic Effect of Cytokine-Induced Killer Cells on Pancreatic Cancer Enhanced by Dendritic Cells Pulsed with K-Ras Mutant Peptide

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Objective. This study is to investigate the role of the CIKs cocultured with K-ras-DCs in killing of pancreatic cancer cell lines, PANC-1 (K-ras$^+$) and SW1990 (K-ras$^-$). *Methods*. CIKs induced by IFN-γ, IL-2, and anti-CD3 monoantibody, K-ras-DCCIKs obtained by cocultivation of k-ras-DCs and CIKs. Surface markers examined by FACS. IFN-γ IL-12 ,CCL19 and CCL22 detected by ELISA. Proliferation of various CIKs tested via 3H-TdR. Killing activities of k-ras-DCCIKs and CTLs examined with 125IUdR. *Results*. CD3$^+$CD56$^+$ and CD3$^+$CD8$^+$ were highly expressed by K-ras-DCCIKs. In its supernatant, IFN-γ, IL-12, CCL19 and CCL22 were significantly higher than those in DCCIK and CIK. The killing rate of K-ras-DCCIK was greater than those of CIK and CTL. CTL induced by K-ras-DCs only inhibited the PANC-1 cells. *Conclusions*. The k-ras-DC can enhance CIK's proliferation and increase the killing effect on pancreatic cancer cell. The CTLs induced by K-ras-DC can only inhibit PANC-1 cells. In this study, K-ras-DCCIKs also show the specific inhibition to PANC-1 cells, their tumor suppression is almost same with the CTLs, their total tumor inhibitory efficiency is higher than that of the CTLs.

1. Introduction

The incidence of pancreatic cancer has shown a clear uptrend [1], and the prognosis in last 20 years has not yet improved [2]. Current immunotherapies for pancreatic cancer mainly include active specific immunotherapy, monoclonal antibody-directed therapy, cytokine therapy, and adoptive cellular immunotherapy [3]. The aim of this study is to investigate the role of the cytokine-induced killer cells (CIKs) cocultured with dendritic cells (DCs) and pulsed with K-ras (12-Val) mutant peptide in the killing of pancreatic cancer cell lines, PANC-1 and SW1990, both in vivo and in vitro.

Dendritic cell (DC) is an antigen-presenting cell, whose function is strongest in the body. They play a role as the bridge and the pivot in the interaction of tumor cells and T lymphocytes [4]. The killer cells induced by cytokines IFN-γ and IL-2 and anti-CD3 monoclonal antibody (cytokine-induced killer, CIK) non-MHC-restrictive cytotoxic T lymphocytes, which kills tumor cells via recognition to a series of related ligands expressed in tumor surface [5, 6]. So far, these cells are considered to have the fastest proliferation, the strongest tumor cytotoxicity, and the most extensive range of tumor killing. Hence, they are the first choice for the adoptive immunotherapy of tumors [7]. Cocultivation of CIK and DC pulsed with K-ras (12-Val) protein peptide, which contains a specific mutation site, can increase the existence of antigen-specific CTL subsets and DC-induced specific CTL activity. Meanwhile, strengthening CIK cell proliferation can further be expected to improve the scope and effects of antitumor immunotherapy. As of now, the research on synergy therapy for pancreatic cancer with the K-ras antigen-allergized DC and CIK has not yet been reported.

2. Materials and Methods

2.1. Materials. rhIL-2 and GM-CSF were purchased from R&D Inc. (USA). IL-4, TNF-4, and IFN-γ were acquired from Peprotech Inc.. Fetal bovine serum (FBS) and cell

medium RPMI1640 were sourced from Sigma-Aldrich Co, Ltd (USA). Lymphocyte separation medium Ficoll and normal human AB serum were purchased from TBD Inc. (Tianjin, China). Mouse anti-human CD3 (FITC labelled) monoclonal antibody, mouse anti-human CD56 (PE labelled) monoclonal antibody, mouse anti-human CD8 (PE labelled) monoclonal antibody, and mouse anti-human CD3 (unlabelled) monoclonal antibody were bought from eBioscience Co, Ltd. Mouse anti-human CD80-PE monoclonal antibody, mouse anti-human CD83-PE monoclonal antibody, mouse anti-human CD86-PE monoclonal antibody, mouse anti-human CD40-FITC monoclonal antibody, and mouse anti-human CD1a-FITC monoclonal antibody were all products of Immunotech Co, Ltd (France). CCL19 and CCL22 ELISA kits were from ADL Inc.. Mouse anti-human Fascin-1 monoclonal antibody and goat anti-mouse IgG secondary antibody were from Santa Cruz Co, Ltd. Cell strains, PANC-1 and SW1990, are available from ATCC. K-ras mutant epitope peptide KLVVVGAVGVGKSALTC was synthesized by SBS Genetech., Ltd.. Female nude mice (BALB/c, 5–8 weeks of age) raised under SPF circumstance were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences.

2.2. Preparation of DCs and CTLs. 50 mL of peripheral blood was sterilely collected from a healthy adult volunteer. Peripheral blood mononuclear cells (PBMCs) were then obtained by lymphocyte separation medium, washed twice with RPMI1640, then diluted to 2×10^6/mL with RPMI1640 containing 10% (V/V) human AB serum. Subsequently, these cells were transferred into culture flasks and cultured for 1-2 hours. Nonadherent cells were harvested as the progenitor of cytokine-induced killer (CIK). The remaining adherent cells were cultured by adding DC medium (containing 0.2 mg/L GM-CSF, 1000 U/mL rhIL-4), and exchanged half amount of DC medium in the next day until 7 days. K-ras mutant epitope peptide was then added into the culture on the 7th day. After 24 hours of cultivation, the culture was induced by adding TNF-α (10 ng/mL) over the following 2 days. Lymphocytes at the final density of 2×10^5/well were then mixed with the k-ras antigen-pulsed DC at 2×10^4/well in 96-well plate, respectively. Under 37°C and 5% CO_2, CTL cells were cultured for 5 days for induction by the specific antigen after which it was ready for use [8].

2.3. CIK Cell Induction and Proliferation. The density of the harvested nonadherent cells was adjusted to 1×10^6/mL with PRMI1640 medium. After adding IFN-γ 1000 U/mL, the culture was cultivated under the condition of 37°C and 5% CO_2 for 24 hours, when CD3 monoclonal antibody (50 ng/mL) and rhIL-2 (1000 u) was added. Subsequently, these cells exchanged half the amount of medium every three days and supplemented CD3 monoclonal antibody and rhIL-2 [9].

2.4. Culture of DCCIKs and Detection for their Cytokine and Proliferation Activity. In 96-well plates, CIKs with density 2×10^5/well were mixed with the antigen-unpulsed DCs and K-ras peptide antigen-allergized DCs, which had been induced and cultured for 9 days, at the density of 2×10^4/well. The cell mixtures were then cultured with CIK medium under the condition of 37°C and 5% CO_2 for 5 days. After adding ^{3}H-TdR (37 kBq/well), these cells were then cultured for another 12 hours. After 12 hours, the cell mixtures were collected and examined by verifying their cpm values with a liquid scintillation counter and by counting their stimulation index (SI): SI = (cpm of experimental group − cpm of background)/(cpm of control group − cpm of background). The proliferation of CIKs, DCCIKs, and K-ras-DCCIKs was observed. Moreover, IL-12 and IFN-γ in the supernatants of the cells cultured for 14 days were tested by ELISA.

2.5. Morphologic Observation and Cellular Phenotype Analysis of DCs and DCCIKs. Morphological changes of DCs and DCCIKs were observed by scanning and transmission electron microscopy after which, the DCs cultured for 7 days and the K-ras pulsed DCs cultured for 9 days were harvested. Using FACS, their phenotype molecules, CD1a, CD80, CD83, CD86, and HLA-DR, were measured and recorded. Afterward, the K-ras-DCs that were originally cultured for 9 days were co-cultivated with CIKs for 5 days. Subsequently, CIKs, DCCIKs, and K-ras-DCCIKs were collected at the 14th day of the cultivation, and the expression of surface markers, CD3, $CD3^+CD56^+$, and $CD3^+CD8^+$, was examined and recorded.

2.6. Detection of CCL19, CCL22, and Fascin-1 of k-Ras-DCCIKs. CIK cocultured with DC and DC pulsed with K-ras peptide at day 9th, the supernatants of the CIK, DCCIK and K-ras-DCCIK were collected at time points of preloading, 6 hours, 12 hours, 24 hours, and 48 hours, respectively. The CCL19 and CCL22 contents (absorbance) in the supernatants were tested separately by ELISA, three repeats for each group. Furthermore, CIKs, DCCIKs, and K-ras-DCCIKs that had been cultured for 14 days were harvested for protein extraction. Fascin-1 protein samples of each group were separated by SDS-PAGE and detected by western blot. β-actin was used as an internal reference. Mouse anti-human Fascin-1 monoclonal antibody was used as primary antibody (1 : 3000), and goat anti-mouse IgG polyclonal antibody was used as secondary antibody (1 : 5000).

2.7. Killing Activity of Different CIKs and CTLs to PANC-1 [10] and SW1990 Pancreatic Cancer Cells. The K-ras-DCCIKs, DCCIKs, CIKs, and CTLs cultured for 14 days were used as effector cells and PANC-1 and SW1990 as target cells. 1×10^6 tumor cells in log phase were collected and added 5 μCi ^{125}I-UdR and final concentration of 5 mol 5-fluorouracil. The cells were incubated in suspension culture under the condition of 37°C and 5% CO_2 for 2 hours. After washing three times with IMDM medium to eliminate the unlabelled ^{125}I-UdR, the tumor cells were counted with γ-counter. Only the cells that had average labelling yield above 1 cpm were used as target cells. Then, the target cells were adjusted with IMDM medium containing 10% FCS to the density of 5×10^5/mL to be ready for use. In accordance with different effector-target ratio (1 : 6.25, 1 : 12.5, 1 : 25, 1 : 50), the effector cells were mixed with the target cells and supplemented with medium to yield one milliliter (three

repeats). Meanwhile, the control group of target cells was used to test the spontaneous release rate. After centrifugation of 1000 r/min for 3 minutes, the effector-target cell mixtures were cultured for 12 more hours. Finally, the cell mixtures were centrifugated at 2000 r/min for 5 minutes and tested for their cpm values. The cytotoxic activity is shown with ^{125}I-UdR release percentage, which is calculated according to the following formula: ^{125}I-UdR release percentage = (cpm value of experimental group − cpm value of spontaneous release group)/(cpm value of maximum release − cpm value of spontaneous release group).

2.8. Animal Experiment. PANC-1 and SW1990 in log phase were prepared to 1×10^7/mL cell suspension. Every female BALB/c nude mice of 5–8 weeks old were subcutaneously inoculated in their backs with 0.2 mL of the suspension to build the tumor-bearing mouse model. At the 10th day after inoculation, the mice were randomly divided into five groups (10 mice per group). Experimental group (I) Group k-ras-DCCIK, k-ras-DCCIKs, were used for every injection. (II) Group DCCIK, DCCIKs were used. (III) Group CIK, CIKs were used. (IV) Group CTL, CTLs induced with k-ras-DCs, were used. (V) Group saline control, saline water was used. 2×10^6 CTLs and various CIKs were injected intratumor every two days, respectively, ten injections in total. Before every injection, the long diameter (L) and the short diameter (S) of the tumors were measured. And tumor sizes were estimated by formula: $V = (L \times S^2)/2$. The final survival time of each group was observed.

2.9. Statistical Analysis. The statistic software SPSS 16.0 was used for data analysis, and Lab-wiok4.6 was used for analysis of western blot results. Measurement data were indicated with mean ± standard deviation. And the original data were tested via homogeneity of variance, and then used for t-test and variance analysis. The differences were deemed to show statistical significance when $P < 0.05$. And the final statistic values of different samples are the gray value ratios of the samples and their relevant internal references.

3. Results

3.1. Morphological Observation of DC and CIK

3.1.1. Morphological Observation of K-ras (12-Val) Mutant Peptide-Pulsed DC. After being pulsed with K-ras (12-Val) mutant peptide, DCs showed larger soma with plenty of dendritic bulges on their surfaces (Figure 1(a)) under scanning microscopy. The DCs also showed irregular shape. From the microscopy, large and long dendritic bulges and small ones were observed on the surface. In the DCs, organelles are abundant. Many mitochondria and rough endoplasmic reticulum were present in the image, though less lysosomes were observed (Figure 1(b)).

3.1.2. Morphological Observation of CIK and DCCIK. Under microscopy, CIKs showed cluster-like growth. And after 3 days of incubation, CIKs' cell masses gradually multiplied and became larger. On the 7th day, the cells began to look rounded with regular shapes. After DCs and CIKs were co-cultured for 14 days, the cells aggregated together to form many cell masses, on the surfaces of which there were plenty of dendritic bulges (Figure 1(c)).

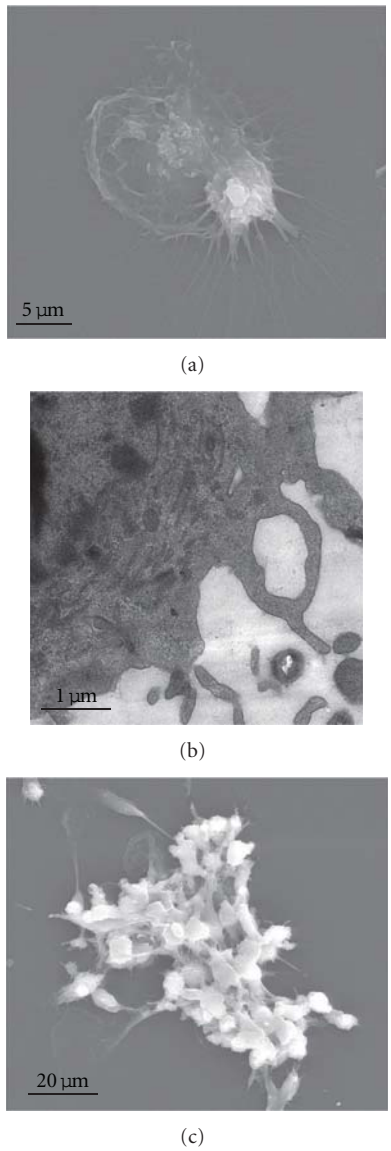


Figure 1: The morphological changes of k-ras pulsed DC and K-ras-DCCIK under scanning and transmission electron microscopy (experiment 2.5). DCs pulsed with K-ras mutant peptide show a larger soma, and plenty of dendritic bulges on their surfaces (Figure 1(a)). Under transmission electron microscopy, DCs show irregular-shape, large, and long dendritic bulges on their surfaces. In the DCs, organelles are abundant, and many mitochondria and rough endoplasmic reticulum can be seen, but less lysosome are present (Figure 1(b)). Coculture of DCs and CIKs for 14 days; under scanning electron microscopy, cells aggregated together to form many cell masses, on the surfaces of which there were plenty of dendritic bulges (Figure 1(c)).

3.2. Cellular Phenotype Detection of DC, CIK, and DCCIK. The expression levels of CD1a, CD80, CD83, and HLA-DR of K-ras-DC were higher than those of the unpulsed DC

Table 1: Surface marker of DCs induced by different antigens (%, $X \pm SD$, $n = 6$).

Groups	CD1a	CD80	CD83	CD86	HLA-DR
DC	22.6 ± 3.6	49.4 ± 3.2	38.5 ± 4.6	72.6 ± 5.6	66.5 ± 4.6
DC(K-ras$^+$ peptide,10 μg/mL)	35.1 ± 4.3	62.2 ± 5.8	51.1 ± 4.9	74.4 ± 5.2	82.4 ± 4.4

Note: DC (K-ras$^+$ peptide, 10 μg/mL) versus DC, $P < 0.05$ except CD86.

Table 2: Surface marker of CIK and DCCIK induced by antigens (%, $X \pm SD$, $n = 6$).

Groups	CD3	CD3$^+$CD56$^+$	CD3$^+$CD8$^+$
CIK	66.34 ± 4.54	34.18 ± 2.63	56.38 ± 4.87
DCCIK	71.4 ± 55.26	39.21 ± 3.12	54.23 ± 4.14
K-ras-DCCIK	87.53 ± 6.02	57.43 ± 4.34	68.65 ± 3.32

Note: DCCIK pulsed with K-ras peptides versus CIK and DCCIK, ($P < 0.05$).

group ($P < 0.05$). However, no significant difference was observed with tCD86 expressions among the groups ($P > 0.05$) (Table 1.). This demonstrated that the dendritic cells can express the mature surface molecules after antigen allergization. After cocultivation, the K-ras-DCCIK population can express CD3$^+$CD8$^+$ and CD3$^+$CD56$^+$ at levels which were significantly higher than those of the unpulsed DCCIK group and the CIK group ($P < 0.05$) (Table 2).

3.3. ELISA Test for Chemokine, CCL19 and CCL22, and Western Blot Analysis for Cytoskeletal Protein, Fascin-1. The CCL19 and CCL22 expression levels in the culture supernatants of group K-ras-DCCIK and group DCCIK were universally higher than those of group CIK except for preloading and the first 6-hour point. Moreover, the CCL19 and CCL22 levels in group K-ras-DCCIK and group DCCIK also showed uptrend with time. After testing at 12 hours, their levels increased more significantly ($P < 0.01$). While at the same time, CCL19 and CCL22 expression levels in group CIK showed no apparent increase. Finally, the comparison of the chemokine expression between group K-ras-DCCIK and group DCCIK also has statistical difference ($P < 0.05$) (Figures 2(a) and 2(b)).

3.3.1. Western Blot Analysis for Cytoskeletal Protein, Fascin-1. The result is shown in Figures 3(a), 3(b), and 3(c), which demonstrates the fascin-1 expressions in DCCIK, CIK, and K-ras-DCCIK (cultured for 14 days). After protein bands analysis with Lab-wiok4.6, it is shown that the expression of the cytoskeletal protein, fascin-1, in K-ras-DCCIK had increased significantly. Compared with group DCCIK and group CIK, the differences showed statistical significance ($P < 0.01$). Furthermore, the comparison between DCCIK and CIK shows that the difference was also significant ($P < 0.05$). The gray values of the reference protein bands are almost equal. This proved that β-actin can be stably expressed in the cells. The results demonstrated that K-ras mutant antigen peptide can facilitate the migration activity of DCCIKs.

3.4. Proliferation Activity Test of CIK and DCCIK. CIKs began proliferating from the third day of culture, and the cell

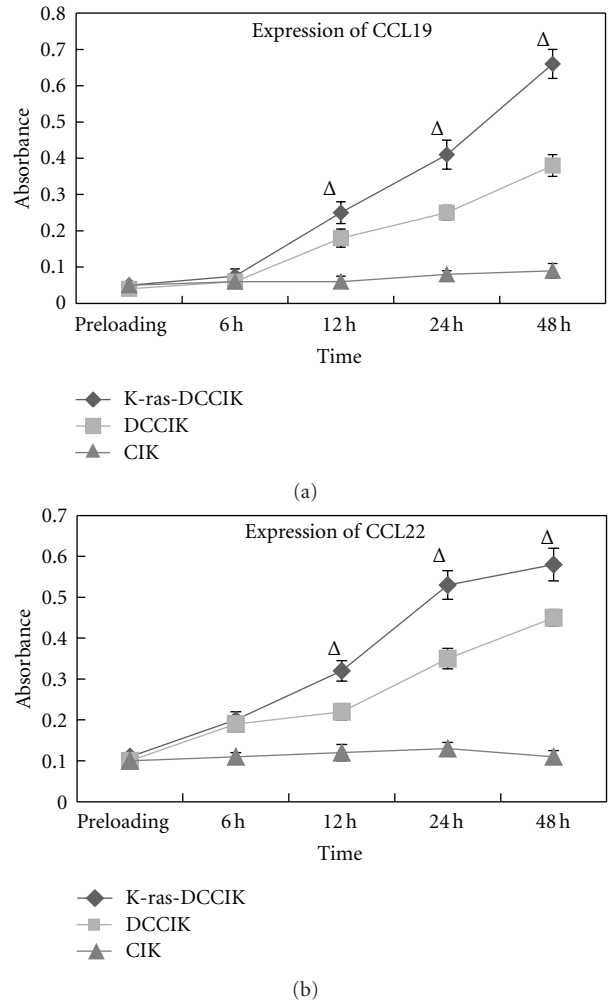


Figure 2: The absorbances of CCL19 and CCL22 at different time points (experiment 2.6). The expression of CCL19 and CCL22 in group K-ras-DCCIK and DCCIK showed uptrend with time and were higher than those of group CIK except for preloading and the 6-hour point. K-ras-DCCIK DCCIK versus CIK after 12 h, $^*P < 0.01$. K-ras-DCCIK versus DCCIK after 12 h, $P < 0.05$. But the expression in group CIK showed no apparent increase (Figures 2(a) and 2(b)).

proliferation sped up on the sixth day with the cell population increasing noticeably. When cultured for 14 days, proliferation capacity of K-ras-DCCIK was remarkably greater than that of other groups ($P < 0.01$). DCCIK proliferation was also greater than CIK ($P < 0.05$) (Figure 4). This showed that K-ras-DC can stimulate the proliferation of CIK effectively. After being allergized by peptide antigen, the DC's ascending secretion of IFN-γ and IL-12 further stimulated the CIK's proliferation.

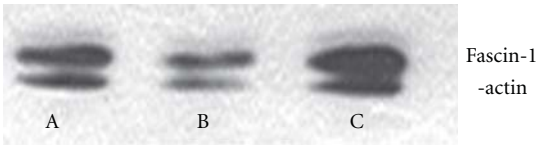


Figure 3: The fascin-1 and β-actin protein expression of DCCIK CIK and K-ras-DCCIK by western blot (experiment 2.6). A, B, and C demonstrate the fascin-1 expression in DCCIK, CIK, and K-ras-DCCIK cultured for 14 days. Compared with group DCCIK and CIK, fascin-1 in K-ras-DCCIK was increasing significantly ($P < 0.01$). DCCIK versus CIK has statistical significance ($P < 0.05$). The gray values of the reference protein bands are almost equal. It was shown that β-actin can be stably expressed in the cells.

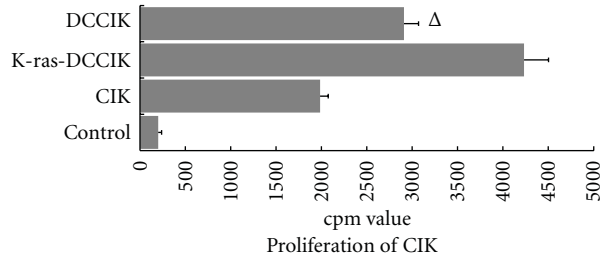


Figure 4: The proliferation activity of CIK cultured with K-ras antigen-pulsed DC (experiment 2.3, 2.4). When cultured for 14 days, proliferation capacity of K-ras-DCCIK was remarkably greater than other groups ($^*P < 0.01$). And DCCIK proliferation was also greater than CIK ($^\Delta P < 0.05$). It is proved that K-ras-DC can stimulate the proliferation of CIK effectively.

3.5. Detection of Cytokine, IL-12 and IFN-γ. In the supernatant of group K-ras-DCCIK cultured for 14 days, IFN-γ and IL-12 levels were higher than those of group CIK and group DCCIK ($P < 0.01$). And IFN-γ and IL-12 levels in the supernatant of group DCCIK were also higher than those of group CIK ($P < 0.05$. After coculture of CIKs and DCs, IFN-γ and IL-12 levels in the cell supernatants can be increased. Furthermore, the antineoplastic activity of the specific antigen-pulsed DCCIK became stronger (Figure 5).

3.6. Detection for Killing Activity of CIK and CTL to PANC-1 and SW1990 Pancreatic Cancer Cell In Vitro. The K-ras-DCCIKs, DCCIKs, CIKs, and CTLs induced by K-ras pulsed DCs were used as effector cells, and the pancreatic cancer cell strains, PANC-1 and SW1990, were used as target cells. The different killing effects of the CIK groups on PANC-1 showed that the killing rate of group K-ras-DCCIK was greatest and significantly exceeded group CIK and group CTL ($P < 0.01$). However, there was no difference between group CIK and group CTL ($P > 0.05$). After increasing the effector-target ratio, the killing rates of the effector cells against the pancreatic cancer cells in all the groups also became higher (Figure 6(a)). As the different killing effects of the CIKs groups on SW1990 demonstrated K-ras-DCCIKs, DCCIKs, and CIKs, all showed their killing effects on SW1990 cells, and their killing rates are higher than group CTL ($P < 0.01$).

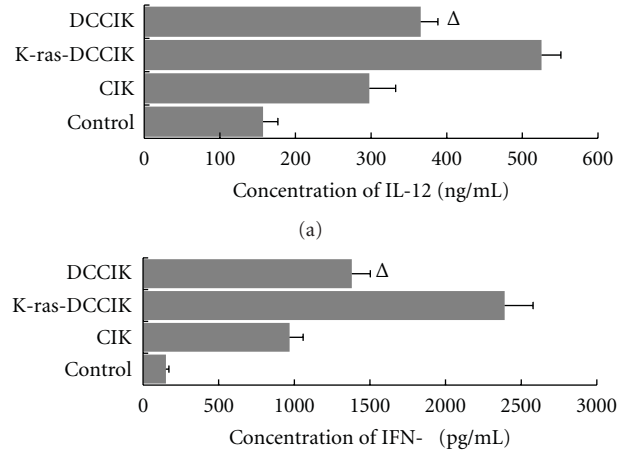


Figure 5: The production of IL-12 and IFN-γ of CIK cultured with K-ras antigen-pulsed DC (experiment 2.4). In the supernatant of group K-ras-DCCIK cultured for 14 days, IFN-γ and IL-12 levels were highest, K-ras-DCCIK versus DCCIK and CIK ($^*P < 0.01$). And levels in group DCCIK were also higher than those of CIK ($^\Delta P < 0.05$). It is demonstrated that after coculture of CIKs and k-ras-DCs, IFN-γ and IL-12 levels in the cell supernatants can be apparently increased.

But the comparison of the killing effect between group K-ras-DCCIK and group DCCIK showed no statistical significance ($P > 0.05$) (Figure 6(b)).

By testing the in vitro killing inhibitions of K-ras-DCCIKs to PANC-1 (K-ras$^+$) and SW1990 (K-ras$^-$), it was found that, when effector-target ratio reached 1 : 12.5 and 1 : 25, K-ras-DCCIKs' inhibition to PANC-1 was stronger than that to SW1990 ($P < 0.05$). However, effector-target ratio increased to 1 : 50; K-ras-DCCIKs' inhibition to these two cells showed no statistical difference ($P > 0.05$) (Figure 7).

3.7. The Effects of Various CIKs and CTL on Survival Time of Tumor-Bearing Nude Mice Loading PANC-1 and SW1990 Pancreatic Cancer Cells. 2×10^6 cells of CIK groups and k-ras-DC-induced CTL group were injected intratumorly into the tumor-bearing nude mice, and their effects on the mice survival time were investigated. Concerning the effects on the survival time of PANC-1 (K-ras$^+$) tumor-bearing mice (Figure 8(a)), the survival time of group K-ras-DCCIK was prolonged remarkably. There is significant difference in comparison with other groups ($P < 0.01$). In group DCCIK, group CIK, and group CTL, the mice survival times were extended correspondingly. But there were no statistical differences among the groups ($P > 0.05$). It is demonstrated that the DC-induced specific CTL can inhibit PANC-1. Meanwhile, K-ras-DCCIK can produce specific and immediate killing effect on PANC-1. This will lead to prolonged survival time. Concerning the effects on the survival time of SW1900 (K-ras$^-$) tumor-bearing mice (Figure 8(b)), the survival time of group K-ras-DCCIK, group DCCIK, and group CIK was elongated dramatically. Also, compared with group CTL, the difference has statistical significance ($P < 0.01$).

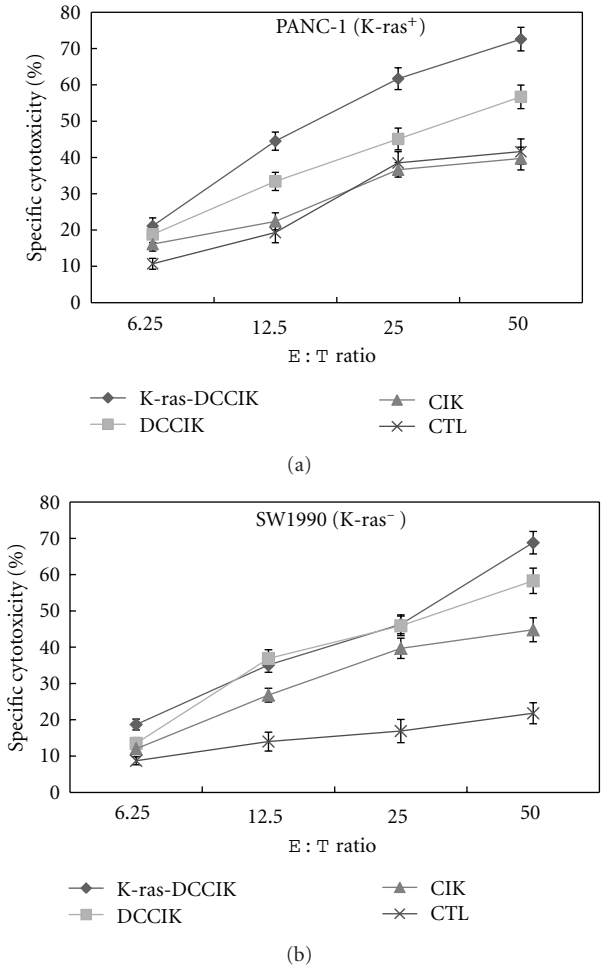


Figure 6: The cytotoxicity of K-ras-DCCIK, DCCIK, CIK, and CTL induced by K-ras pulsed DC against PANC-1 (K-ras$^+$) and SW1990 (K-ras$^-$) cells in vitro (experiment 2.7). The K-ras-DCCIKs, DCCIKs, CIKs, and CTLs induced by K-ras pulsed DCs used as effector cells. PANC-1 and SW1990 used as target cells. The killing effects on PANC-1 showed that group K-ras-DCCIK exceeded group CIK and group CTL remarkably ($P < 0.01$). However, there was no difference between group CIK and CTL ($P > 0.05$), (Figure 6(a)). The different killing effects on SW1990 demonstrated K-ras-DCCIKs, DCCIKs, and CIKs; all showed their killing effects on SW1990 cells and are higher than CTL ($P < 0.01$), (Figure 6(b)).

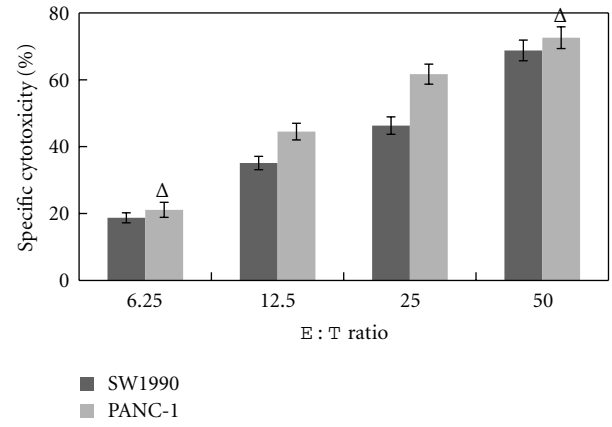


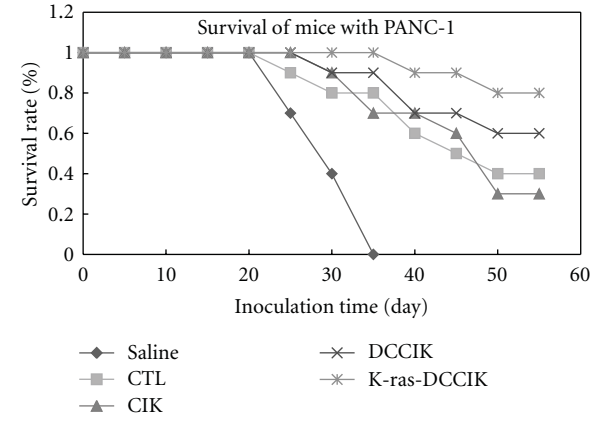
Figure 7: The cytotoxicity of K-ras-DCCIK against PANC-1 (K-ras$^+$) and SW1990 (K-ras$^-$) cells at different $E : T$ ratio in vitro (experiment 2.7). It was found that when effector-target ratio reached 1 : 12.5 and 1 : 25, K-ras-DCCIKs' inhibition to PANC-1 was stronger than that to SW1990 ($^*P < 0.05$). However, $E : T$ ratio at 1 : 6.25 and 1 : 50, K-ras-DCCIKs' inhibition to these two cells showed no statistical difference ($^\Delta P > 0.05$). It is demonstrated that CIK cells have a potential to uptake tumor antigens, and further, to produce specific killing effect on PANC-1, there might be some antigen-specific CTL cell subsets existing in K-ras-DCCIKs.

However, there were still no significant differences among the groups ($P > 0.05$). It is shown that to varying degrees, the CIK groups possess the direct inhibition to SW1900. In contrast, the specific CTL induced by K-ras mutant peptide-pulsed DC showed the lower inhibition to K-ras mutation-negative cell, SW1900. In group CTL, the survival time of the SW1900 tumor-bearing mice was not extended.

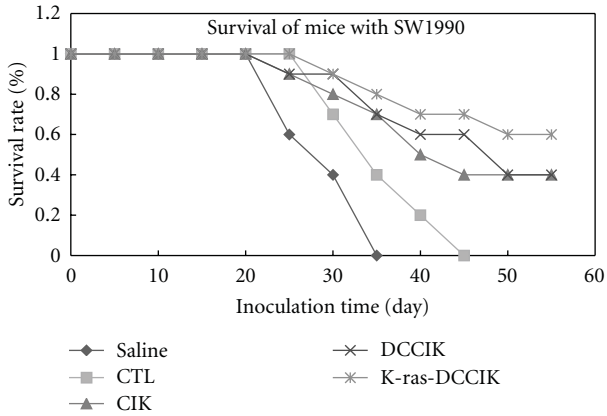
4. Discussions

Tumorigenesis is a sustained process of gene mutations. Almoguera et al. [11] first reported the point mutation of K-ras gene in pancreatic cancer sufferers. Since, it had been investigated that, in 85%–95% of pancreatic cancer patients, K-ras gene mutations occurred and almost all the mutations happened at the 12th codon. Hence, the 12th mutational site of K-ras protein can be used as a potential site for gene immunotherapy of pancreatic cancer [12, 13]. Nakada et al. [14] and others had used the antisense oligonucleotides for K-ras gene mutations to transfect the pancreatic cancer cell, PANC-1. This treatment can inhibit the mRNA expression of K-ras gene and the synthesis of ras protein. Thus, it can suppress the growth of pancreatic cancer cell and facilitate the apoptosis of cancer cells. He et al. [15] and others had tried to use K-ras mutated peptide to modify DCs in order to activate T cells. It had been found that DCs can present K-ras mutated sites effectively. In this study, we used K-ras mutated peptide to modify DCs. After coculture with the modified DCs, CIKs showed immediate and specific inhibition to pancreatic cancer cells in vitro and in vivo.

CIKs is a cell population obtained from human peripheral blood mononuclear cells stimulated with IFN-γ, IL-2, and CD3 monoclonal antibody (OKT3). They can express the surface markers of T cells and NK cells, CD3$^+$CD56$^+$ [16]. For now, CIK is known to have the fastest proliferation, the strongest tumor cytotoxicity, and the most extensive range of tumor killing [17]. CIKs' killing action to tumors is via recognition of a series of associated ligands on tumor cell surfaces, though not only depending upon one antigen [5]. CIKs can both directly inhibit tumor cells and regulate immune system of body to kill tumor cells indirectly [18]. Therefore, they can suppress the tumor's growth and recurrence by immediate inhibition to tumor cells and improve the immunity of patients for long-term effect [19]. CIKs seldom arouse graft-versus-host disease (GVHD) and are safe and effective for patients who had developed drug resistance.



(a)



(b)

Figure 8: Survival of nude mice inoculated subcutaneously in the back with PANC-1 (K-ras$^+$) and SW1990(K-ras$^-$) cells after immunotherapy with CTLs and different CIKs (experiment 2.8) concerning the effects on the survival time of PANC-1 (K-ras$^+$) tumor-bearing mice (Figure 8(a)), the survival time of group K-ras-DCCIK was prolonged remarkably, compared with other groups ($P < 0.01$). No statistical difference was found among the group DCCIK, CIK and CTL ($P > 0.05$). It is demonstrated that the k-ras-DC induced CTL can inhibit PANC-1. Meanwhile, K-ras-DCCIK can produce specific and immediate killing effect on PANC-1. Concerning the effects on the survival time of SW1900 (K-ras$^-$) tumor-bearing mice (Figure 8(b)), the survival time of group K-ras-DCCIK, DCCIK and CIK was elongated dramatically. Compared with group CTL ($P < 0.01$). The CIK groups possess the direct inhibition to SW1900. The CTL induced by K-ras-DC showed the lower inhibition to K-ras mutation negative cell, SW1900. Thus, the survival time of the SW1900 tumor-bearing mice was not extended.

Coculture of CIKs with DCs can increase their proliferation activity and cytotoxicity [20]. At present, the mechanism of why DCs can enhance CIKs' killing activity is still unclear. It has been speculated that the strengthened tumor-killing effect of DCCIKs may be associated with upregulation of cytokines, such as IL-12 and IFN-γ, in DCCIKs' supernatant and with high expression of CD3$^+$CD56$^+$ double positive cells as well [21]. In this study, by testing the expressions of DCs' surface molecules, the results show that K-ras mutated peptide can promote DCs' mature and facilitate effective presentation of specific antigens [22]. IFN-γ and IL-12 levels of group K-ras-DCCIK are highest, superior to those of group DCCIK and group CIK remarkably ($P < 0.01$). Moreover, after co-cultivation, K-ras-DCCIKs highly expressed CD3$^+$CD8$^+$ and CD3$^+$CD56$^+$, exceeding those of group CIK significantly ($P < 0.05$).

The chemokine, CCL19, is expressed in secondary lymphoid organs and thymus. It can urge DCs to migrate from peripheral region to T-cell accumulation area in lymphoid organs and induce Th1 and T cells to make an immune response [23–25]. CCL22 is expressed in the spleen, peripheral blood T cells, NK cells, and so on. [26]. In the supernatant of the monocyte-derived DCs, intact CCL22 become highly expressed and produce intense chemotaxis for DCs [27, 28]. In ELISA test for CCL19 and CCL22 of various CIK groups, it was founded that k-ras-DCs can apparently enhance CIKs' migration activity and can improve their migration capacity towards tumor cells. These actions provide some necessary conditions for increasing killing activity and suppressing tumor growth. In immune system, Fascin-1 protein is only expressed in the mature DCs and related to DCs' movements [29, 30]. The research indicated that the secretory volume of k-ras-DCCIKs' cytoskeletal protein Fascin-1 was increased remarkably. Compared with group DCCIK and group CIK, the increasing has dramatically statistical significance ($P < 0.01$). It was demonstrated that K-ras mutated peptide can induce DCs' mature and enhance k-ras-DCCIKs' migrating capacity.

k-ras-DCCIKs' proliferation capacity is significantly higher than that of other groups ($P < 0.01$). It can be seen that k-ras-DC can effectively stimulate CIKs' proliferation. Moreover, the increased secretion of IFN-γ and IL-12 further irritated CIKs' proliferation. Marten and so forth [31] used CA19-9 antigen-pulsed mature DCs and antigen peptide-untreated DCs to coculture with CIKs. In contrast with untreated DCs group, the former showed increased killing activity. It was prompted that, in the CIKs cocultured with CA19-9 pulsed DCs, there were existing antigen-specific CTL subsets. In our in vitro experiment, the killing activity of K-ras-DCCIK group to PANC-1 cells was also superior to those of CIK group and k-ras-DC-induced CTL group ($P < 0.01$). It indicates that, via K-ras pulsing, the DCs further enhanced CIKs' killing activity. Compared with k-ras-DC-induced specific CTLs, CIKs have almost identical killing efficiency ($P > 0.05$). The specific CTLs have obvious inhibition to K-ras$^+$ PANC-1, while K-ras-DCCIKs have immediate PANC-1 killing effect, which is specific and more remarkable so as to make the mice's survival period significantly prolonged. All of the K-ras-DCCIKs, DCCIKs, and CIKs showed inhibitions to SW1990 cells. But k-ras-DC-induced specific CTLs present the weakened suppression towards SW1990 cells. Compared with DCCIKs' inhibition, there is a significant statistical difference ($P < 0.01$). It was shown that, to varying degrees, the CIK groups possess the direct inhibition to SW1900. In contrast, the specific CTLs showed the lower inhibition to K-ras mutation-negative cell, SW1900. In group CTL, the survival time of the SW1900 tumor-bearing mice was not extended. In this study, by co-culturing tumor antigen-pulsed DCs with CIKs, we obtained

the k-ras-DCCIKs, which have more prominent oncotherapy effect and more powerful tumor inhibition than CIKs and show a favourable application prospect.

PANC-1 and SW1990 cells were inhibited with k-ras-DCCIK separately. The results show that, when effector-target ratio reached 1 : 12.5 and 1 : 25, K-ras-DCCIKs can produce specific inhibition to PANC-1. Further, the killing efficiencies towards these two pancreatic cancer cells have statistical difference ($P < 0.05$). However, increasing effector-target ratio, the difference of specific tumor inhibition for these two cells showed no statistical significance ($P > 0.05$). It is demonstrated that CIK cells have a potential to uptake tumor antigens and further to produce specific killing effect on cancer cells. In K-ras-DCCIKs, there might be some antigen-specific CTL cell subsets existing. Allergization for CIKs with tumor antigen-pulsed DCs can both exert non-MHC restrictive cytotoxicity of CIKs and activate MHC restrictive cytotoxicity mediated by antigen-pulsed DCs to strengthen the specific killing effect on specific target cells [32]. However, when effector-target ratio is high, it might be CIKs' strong tumor direct killing effect that covers their specific action. This phenomenon is to be further investigated in the future.

In conclusion, the results demonstrate that, after being pulsed with K-ras, DCs can enhance CIKs' proliferation and migration capacities, and can enhance killing activity against pancreatic cancer cells as well. Moreover, CIKs' enhanced killing activity may be associated with upregulation of IFN-γ and IL-12 in supernatants and high expression of double-positive cells $CD3^+CD8^+$ and $CD3^+CD56^+$. The antigen-allergized DCCIKs can produce in vitro killing activity specific to tumor cells. Their pertinence of tumor suppression is almost the same as with the specific CTLs, while their total tumor inhibitory efficiency is higher than the CTLs.

Abbreviations

CTLs: Cytotoxic T lymphocytes
DCs: Dendritic cells
GM-CSF: Granulo-macrophage-stimulating factor
IL: Interleukin
CIK: Cytokine-induced killer
DCCIKs: CIK cells cocultured with DCs
CD1a, CD80, CD83, CD8, and HLA-DR: DC surface markers
$CD3^+CD8^+$ and $CD3^+CD56^+$: CIK surface markers
Fascin-1: A kind of cytoskeletal protein
PANC-1: The pancreatic cancer cell line with point mutations of K-ras
SW1990: The pancreatic cancer cell line without point mutations of K-ras
SI: Stimulating index
TNF-α: Tumor necrosis factor
PBS: Phosphate-buffered saline
IFN-γ: Interferon
FCS: Fetal calf serum
ELISA: Enzyme-linked immunosorbent assay.

Conflict of Interests

The authors declare that they have no competing interests.

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Review Article

Challenges in Immunotherapy Presented by the Glioblastoma Multiforme Microenvironment

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Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in adults. Despite intensive treatment, the prognosis for patients with GBM remains grim with a median survival of only 14.6 months. Immunotherapy has emerged as a promising approach for treating many cancers and affords the advantages of cellular-level specificity and the potential to generate durable immune surveillance. The complexity of the tumor microenvironment poses a significant challenge to the development of immunotherapy for GBM, as multiple signaling pathways, cytokines, and cell types are intricately coordinated to generate an immunosuppressive milieu. The development of new immunotherapy approaches frequently uncovers new mechanisms of tumor-mediated immunosuppression. In this review, we discuss many of the current approaches to immunotherapy and focus on the challenges presented by the tumor microenvironment.

1. Introduction

Glioblastoma multiforme (GBM) (WHO grade IV astrocytoma) is the most common and malignant primary brain tumor in adults. Despite aggressive, multimodal treatment with maximal surgical resection followed by temozolomide and radiation, the prognosis for patients with GBM remains grim with a median survival of 14.6 months and a 3-year survival rate of only 10% [1]. One formidable challenge in advancing GBM therapy is the complexity of the GBM microenvironment [2]. Elucidating the details of GBM resistance to traditional therapies requires consideration not only of the intrinsic properties of tumor cells, but also how these cells interact with neural precursor cells, tumor stem cells, vascular endothelial cells, stromal cells, astrocytes, microglia, lymphocytes, extracellular matrix proteins, and cytokines. It is this dynamic interplay among diverse cell populations, cytokines, and extracellular matrix proteins that coordinates GBM tumorigenesis, growth, and invasion. Effective therapies, therefore, must not only be directly cytotoxic to a molecularly diverse population of tumor cells [3], but must also overcome the protumorigenic properties of the GBM microenvironment.

Immunotherapy is a particularly attractive approach to cancer treatment as it affords the advantages of cellular level specificity and the potential for generating long-term immune surveillance against cancer cells. The notion of activating the immune system against cancer has been around for decades but has recently come to the forefront with the FDA approval of the first therapeutic cancer vaccine for the treatment of metastatic, castration-resistant prostate cancer [4]. More recently, ipilimumab, an anti-CTLA-4 antibody, was approved by the FDA for first- and second-line treatment of unresectable or metastatic melanoma [5]. Preclinical research is rapidly identifying new immunological targets leading the way for the development of powerful combination therapies [6]. In addition, several immunotherapies are currently in clinical trials and many are producing encouraging results in a variety of cancers [7].

Immunotherapy for neoplasms of the central nervous system (CNS) has been hampered by the traditional belief that the CNS is immunologically privileged [8]. This theory was based on reports of a paucity of native antigen-presenting cells (APCs) in the CNS, the lack of a traditional lymphatic system, impermeability of the blood-brain barrier (BBB) to antibodies and lymphocytes [9], low baseline levels

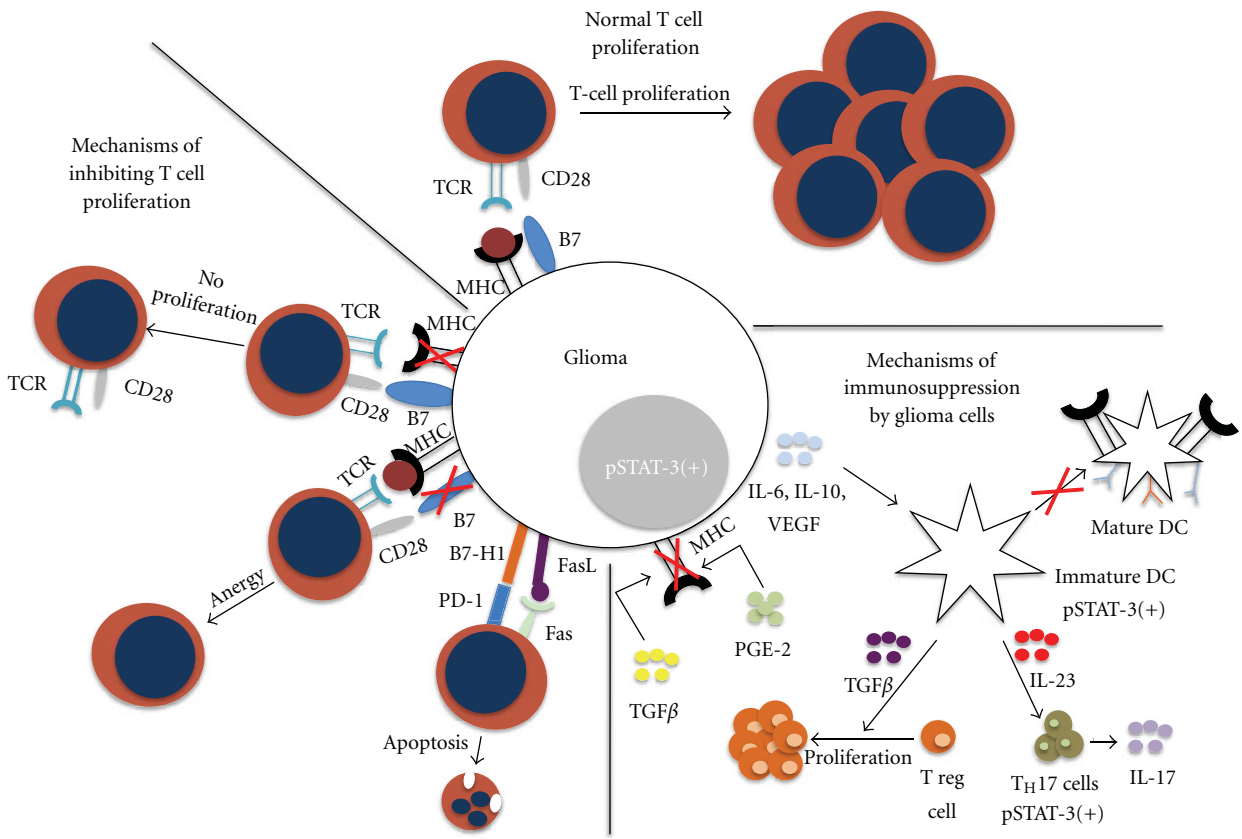

Figure 1: Normal T cell proliferation and mechanisms of glioma cell immunoresistance. (From top moving clockwise) *Normal T cell proliferation:* tumor cell antigens are presented by MHC and costimulatory molecules. *Mechanisms of immunosuppression:* glioma cells secrete factors leading to an immunosuppressive tumor microenvironment. TGFB and PGE-2 downregulate the expression of MHC, restricting antigen presentation and T cell proliferation. IL-6. IL-10 and VEGF are potent STAT-3 activators, leading to the proliferation of immature DCs that are not able to function as APCs. These immature DCs also secrete TGFB which aid in the proliferation of immunosuppressive Treg cells and STAT-3 positive TH17 cells. *Mechanisms of inhibiting T cell proliferation:* glioma cells downregulate MHC on their surface leading to the decreased antigen presentation and decreased T cell proliferation. Downregulation of B7 works via a similar mechanism in that the costimulatory signal is lost preventing T cell proliferation. Increased expression of B7-H1 and FasL act as proapoptotic signals for T cells.

of major histocompatibility complex (MHC) expression [10], altered expression of T cell costimulatory molecules [11], and the observation that tissues engrafted into the CNS are rejected more slowly than those grafted to other sites [12, 13]. Each of these perceived impediments to immunotherapy has subsequently undergone major revisions. Microglia [14], macrophages, and dendritic cells [15, 16] act as powerful APCs in the CNS. Antigens originating within the CNS drain in the cerebrospinal fluid through Virchow-Robin perivascular spaces to nasal and cervical lymph nodes where they can be accessed by naïve T cells [17, 18]. Subpopulations of activated T cells expressing integrins, which impart CNS tropism, such as $\alpha 4\beta 7$, traverse the BBB [19] where they can act as cytotoxic or helper T cells based on CD8 or CD4 expression, respectively [20]. There is also evidence to suggest that naïve T cells traffic to the CNS, especially when inflammation locally increases the permeability of the BBB [21]. Furthermore, antibodies have been isolated from the brain, albeit in much lower concentrations than in plasma [22, 23]. Taken together, these findings represent an evolution in our understanding of the interactions between the CNS and the immune system.

This paradigm shift has generated enthusiasm for a potential role for immunotherapy in GBM. Despite encouraging results in rodent models, however, clinical trials of immunotherapy for GBM have been largely disappointing to date. One of the primary impediments to developing effective immunotherapies is the aforementioned complexity of the GBM microenvironment (Figure 1). Immunosuppressive cytokines such as prostaglandin E2 (PGE-2), TGF-β, and IL-10 are known to be highly expressed in GBMs [24, 25]. In addition, tumor-infiltrating T cells have been shown to exhibit an enriched population of CD4+, CD25+, FoxP3+ regulatory T cells (Tregs) [26]. Expression of the signal transducer and activator of transcription 3 (STAT3) is upregulated in GBM and is believed to promote immunosupression and serve as a point of convergence for several protumorigenic pathways [27]. Furthermore, tumor stem cells have been shown to be immunosuppressive in GBM [28]. Immune checkpoints, such as programmed cell death

Table 1: Selected clinical trials using cytokine modulation.

Reference	Patients	Cytokine	Immunologic response	Clinical response
[58]	$n = 145$ (GBM: 103, AA: 42)	TGF-β	—	Median survival: 39.1 mo (10 uM dose) and 35.2 mo (80 uM dose)
[63]	$n = 9$ Recurrent GBM	IL–2	—	Enhancement of tumor on MRI unchanged (6/9)
[64]	$n = 9$ (GBM: 7, AA: 2)	IL–2	—	PR: 1
[65]	$n = 12$ recurrent GBM	IL–2	Increased inflammatory infiltrate in biopsied tumors	PR: 2, SD: 4, Minor response: 4, Overall survival 58% (6 mo) and 25% (1 yr)
[69]	$n = 31$ (GBM: 26, AA: 5)	IFN-γ ($n = 14$)	—	PR: 3 (Treatment group), No difference in median survival between treatment and control groups
[70]	$n = 40$ (GBM: 14, AA: 14, Other: 12)	IFN-γ	—	No difference in median overall survival
[71]	$n = 29$ (AA: 12, Other: 17)	IFN-β	—	PR: 2, SD: 2
[72]	$n = 20$ (GBM/AA: 15)	IFN-β	IFN-β treatment showed no growth suppression in *ex vivo* assays	SD: 3
[73]	$n = 7$ (GBM: 6, Recurrent AA: 1)	IFN-β	—	No response
[75]	$n = 35$ recurrent HGG	IFN-α	—	Median survival: 13.3 mo
[76]	$n = 275$ HGG	IFN-α	—	No difference in survival
[77]	$n = 9$ (GBM: 6, AA: 2, Other 1)	IFN-α	—	CR: 2
[80]	$n = 12$ (GBM: 11, AA: 1)	IL–4	Positive Elispot assay	No difference in progression free survival
[142]	$n = 9$ recurrent GBM	IL–4	—	Survival > 18 mo ($n = 1$)
[84]	$n = 15$ (GBM: 6, AA: 7, Other: 2)	IL–12	—	PR: 4, Mixed response: 1

1 (PD-1) and Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) may also be manipulated by GBM to induce T cell exhaustion [26, 29]. Finally, there is evidence to suggest that the GBM microenvironment may divert CD4+ T cell differentiation away from a tumor-directed cytotoxic Th1-mediated response and toward a Th17-mediated chronic inflammatory response [30], which has been shown to be protumorigenic in other cancers [31].

Identification of appropriate tumor antigens and generation of a strong antitumor immune response against such a molecularly heterogeneous neoplasm [32] poses a considerable challenge. This challenge is amplified by the immunosuppressive tumor microenvironment. Here, we review the current approaches in immunotherapy for GBM, focusing specifically on how each approach is affected by the array of challenges presented by the tumor microenvironment.

2. Current Approaches

2.1. Cytokine Modulation. Immune responses in the CNS exhibit a distinct hierarchy skewed toward antibody responses and Th2 T cell differentiation [33–35]. It is believed that this hierarchy is maintained by the CNS cytokine milieu [35]. In the GBM microenvironment, the antitumor immune response is further suppressed by high levels of circulating immunosuppressive cytokines such as IL-10, TGF-β, and PGE2 as well as membrane-bound proteins such as FasL and B7-H1 (PD-L1) [36, 37]. The sources of these molecules and the details of their interactions are yet to be fully elucidated. It is clear, however, that the cytokine milieu plays a critical role in coordinating immunosupression in GBM. Clinical trials using cytokine modulation are summarized in Table 1.

2.1.1. TGF-β. TGF-β is synthesized in a pre-pro-TGF-β form and undergoes homodimerization and cleavage by the convertase family of endopeptidases [38] to produce a C-terminal mature peptide and an N-terminal latency-associated peptide, which collectively form the small latency complex [39]. The small latency complex is then secreted from the cell and associates with specific binding proteins to form the large latency complex, which is bound by components of the extracellular matrix [39, 40]. TGF-β is activated when it is released from the latency-associated peptide through one of a number of context-dependent mechanisms [41]. Activated TGF-β regulates gene expression downstream via the SMAD family of transcription factors [39]. TGF-β synthesis, secretion, and signaling are reviewed in detail elsewhere [42].

TGF-β promotes immunosuppression in GBM by inhibiting T cell activation and proliferation, blocking IL-2 production, suppressing activity of NK cells, and promoting Treg activity [43, 44]. In addition, TGF-β is believed to promote tumor growth and invasion by sustaining GBM stem cells [45], promoting angiogenesis [46], and upregulating expression of molecules such as MMP-2, which are associated with tumor invasion [47]. The involvement of TGF-β in multiple tumorigenic pathways makes this cytokine an enticing target for immunotherapy.

TGF-β expression is increased by radiation both *in vitro* [48] and *in vivo* [49]. This finding is of interest because radiation therapy is a critical component of the tripartite treatment approach of resection, temozolomide, and radiation which has become standard of care for patients with GBM [1], and because there is emerging evidence to suggest that radiation therapy may alter several components of the immune microenvironment [50–52]. Radiation-induced activation of TGF-β is believed to be mediated by reactive oxygen species (ROS), which have been shown to convert latent TGF-β preferentially to the TGF-β1 isoform [53]. Although this isoform plays a more minor role in GBM pathogenesis than the TGF-β2 isoform, available evidence suggests that TGF-β1 promotes immunosuppression [54] and acts as a mediator of radiation-induced DNA damage sustained by nontargeted cell populations [55]. In addition, TGF-β2 has been shown to increase tumor invasiveness by upregulating MMP-2 expression in glioma cells [56] and evidence from other cell lines suggests that TGF-β1 may be an even more powerful inducer of MMP-2 expression [57].

The results of TGF-β blockade in preclinical models have been generally promising. The TGF-β2 antisense oligonucleotide trabedersen (AP12009) has been shown to decrease tumor cell proliferation, inhibit migration, and enhance the antitumor immune response *in vitro*. A randomized, phase IIb clinical trial of trabedersen reported significantly improved tumor control and a trend toward increased 2-year survival for patients with anaplastic astrocytoma as compared with standard chemotherapy (temozolomide or a combination of procarbazine, lomustine, and vincristine) [58]. This trial did not report improved survival in patients with GBM, although a subgroup analysis of young patients with good performance status indicated a trend toward improved 2- and 3-year survival rates. Of note, the reported rate of treatment-related adverse events was approximately 20% higher with standard chemotherapy than with trabedersen. Trabedersen is currently in phase III clinical trials for anaplastic astrocytoma [59]. Understanding the role of TGF-β in the tumor microenvironment may have implications for standard therapies as well. For example, given that available evidence points toward a protumorigenic role for TGF-β, the addition of TGF-β blockade to adjuvant radiation therapy may prove prudent [60].

2.1.2. IL-2. IL-2 is a proinflammatory cytokine which promotes T cell activation and Th1 differentiation while abrogating the immunosuppressive effects of TGF-β [61]. IL-2 therapy for GBM is complicated by the fact that high systemic doses of IL-2 are required to reach therapeutic concentrations in the CNS [62]. Early trials of IL-2 alone or in combination with IFN-α [63] or lymphokine-activated killer (LAK) cells [64] attempted to obviate the severe side effects associated with systemic high-dose IL-2 therapy by delivering IL-2 intratumorally or intraventricularly; however, the patients in these trials experienced significant adverse events resulting from local edema. A more recent trial by Colombo et al. used a retroviral vector and intratumoral implantation of retroviral-producing cells to deliver combination HSV-TK/IL-2 gene therapy followed by administration of acyclovir to 12 patients with recurrent gliomas [65]. This trial reported no major adverse events and a radiographic response rate of 50%. Evidence from preclinical models additionally suggests that IL-2 therapy generates long-lasting immune surveillance, which is capable of eliminating tumor cells both inside and outside the CNS [66]. Current approaches to IL-2 therapy for GBM are focused on combination therapy and strategies for local delivery [67].

2.1.3. Interferons. Interferons are secreted by immune cells in response to viruses or other challenges and serve to coordinate the immune response. Alpha interferon (IFN-α), beta interferon (IFN-β) and gamma interferon (IFN-γ) have been extensively studied in cancer immunotherapy. These type 1 interferons have specifically been implicated in coordinating an antitumor immune response against GBM. A study by Fujita et al. demonstrated that mice deficient in type 1 interferons, and induced to develop gliomas *de novo* via p53 knockdown, exhibited enriched populations of tumor infiltrating myeloid-derived suppressor cells and Tregs as well as a decrease in the numbers of tumor-infiltrating CD8+ T cells [68]. Despite some preclinical evidence for efficacy against gliomas, small clinical trials using IFN-γ have been generally disappointing [69, 70]. Trials of IFN-β have produced mixed results [71–73]. The efficacy of IFN-β in combination with temozolomide is currently being investigated [74].

Of the type 1 interferons, IFN-α has been the most extensively studied in GBM. In a phase III study by Buckner et al., 214 patients were initially treated with BCNU and radiation. Patients with radiographically stable disease were subsequently randomized to treatment with a second course

of BCNU or BCNU and IFN-α. This study demonstrated no difference in survival or tumor response with the addition of IFN-α [75]. Unfortunately, there was a significantly increased incidence of side effects, including fever, chills, myalgias, somnolence, confusion, and exacerbation of neurologic deficits in patients receiving IFN-α. These findings were in contrast with a prior phase II study by the same group, which reported that IFN-α was associated with radiographic evidence of tumor regression in 29% of patients and limited toxicity [76]. A more recent trial of IFN-α in combination with local BCNU delivery in patients with recurrent GBM reported 6-month progression-free survival in 2/9 patients [77]. Of interest, both patients who responded in this study were in the group receiving the lowest dose of IFN-α. Therefore, while grade 2 and grade 3 toxicities were observed somewhat frequently in the higher dose groups, only two grade 2 events and no events grades 3 or higher were observed in the treatment group containing the two responders.

2.1.4. Miscellaneous Cytokines. Many cytokines have been evaluated for their effectiveness in GBM therapy. TNF-α knockout mice implanted with GL261 glioma cells have been shown to harbor a decreased number of tumor-associated macrophages and exhibit shorter survival [78]. Knowledge of the role of TNF-α in human gliomas, however, is limited. IL-4 has been shown to increase in CD8+ tumor-infiltrating T cells in a rat model [79]. In a small clinical trial by Okada et al., patients received vaccinations of autologous glioma cells and fibroblasts retrovirally transfected with TFG-IL4-Neo-TK [80]. Treatment was well tolerated, but there was no observed progression-free survival benefit. Locally delivered IL-12 in preclinical models increases tumor-directed T cell responses [81], improves survival, and produces variable development of durable immune surveillance [82]. Tumor stem cells secreting IL-12 have also been shown to track migrating glioma cells and prolong survival [83]. Limited evaluation of IL-12 therapy in clinical trials, however, has produced mixed results [84]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) promotes a CD8+ cytotoxic T cell response when combined with antitumor vaccines [85]. GM-CSF is currently being used as an adjuvant in a phase II vaccination study of patients with newly diagnosed GBM [86]. Discovery of T cell populations producing IL-17 (Th17) [87] and their association with STAT3 expression in human cancers [88] have recently generated an interest in defining the role of these cells in GBM pathogenesis. Early preclinical studies indicate IL-17 is expressed in GBM, but the significance of IL-17 expression in the tumor microenvironment is yet to be clearly defined [30].

2.2. Cellular Immunotherapy. Transfer of *ex vivo* matured immune cells is showing promising results as a future immunotherapeutic intervention against malignant glioma. Initially used as a treatment for melanoma, this strategy involves infusion of autologous immune cells that were matured *ex vivo* with activity specific for glioma cell antigens. While studies have shown lymphokine-activated killer cells cannot effectively migrate across the BBB, effector T cells are able to cross the BBB allowing for a vaccine or IV strategy to be used [89].

2.2.1. Lymphokine Activated Killer Cells. Lymphokine activated killer (LAK) cells are autologous peripheral blood lymphocytes that have been stimulated *in vitro* with IL-2 [90]. Results of early clinical trials infusing LAK cells directly into the surgical cavity showed promise for the use of LAK cells as an immunotherapeutic strategy [64, 91, 92]. The most encouraging of these early studies, Hayes et al. reported a median survival in 18 patients of 12.2 months compared with the control group of 6.2 months with minimal toxicity [93]. In 2004, Dillman et al. reported minimal toxicity and an increase in median survival in a trial of 31 patients. Median survival from the date of original diagnosis was 17.5 months versus 13.6 months for a control group of 41 contemporary GBM patients [94]. Of note, LAK cells must be administered directly to the tumor site since they fail to effectively migrate from the periphery into the brain [95]. Clinical trials using LAKs are summarized in Table 2.

2.2.2. E ector T-Cells. Effector T cell therapy involves transfer of autologous cytotoxic T cells (CTLs) specific for tumor antigens, which are matured from peripheral blood mononuclear cells (PBMCs) or T cells from the tumor itself, to the host. This therapy is based on the theory that T cells can migrate to the site of a tumor by crossing the BBB, and selectively exert cytotoxic effects on tumor cells. This strategy has been studied extensively in malignant melanoma with promising results. Studies in animal models of glioma have been promising. Initial studies by Yamasaki and Kikuchi used IL-2 to activate CD8+ T cell clones with target specificity against murine malignant brain tumor cells. This strategy resulted in successful migration of T-cells to the tumor, cytotoxic activity against the tumor, and a significant increase in survival after IV infusion [96].

Early clinical studies using *ex-vivo*-expanded CTLs were largely disappointing for patients with GBM, however, more recent studies have shown promise. Tsurushima et al. reported that activating polyclonal T cells with IL-2 resulted in two patients with Grade III disease exhibiting complete tumor regression for at least 5 years with another patient having a partial regression [97]. A study using GM-CSF resulted in three of ten patients having at least partial tumor regression. All patients with a diagnosis of GBM survived at least one year from the time of adoptive transfer [98]. Another approach has been to genetically modify T cells to express a chimerical antigen receptor (CAR) for a known tumor antigen. Kahlon et al. genetically engineered CD8+ T cells to express CARs for IL-13Rα2 and reported regression of GBM xenografts [99]. Studies in human GBM have demonstrated that CARs can migrate to tumors *in vivo* [100]. Furthermore, Ahmed et al. have shown that CARs targeted to HER2 are able to eliminate CD133+ stem cells as well as bulk tumor cells in HER2+ GBMs [101]. Clinical trials using CTLs are summarized in Table 3.

Table 2: Selected clinical trials using lymphokine activated killer (LAK) cells.

Reference	Patients	Immunologic response	Clinical response
[64]	$n = 9$ (GBM: 7, AA: 2)	—	PR: 1
[91]	$n = 9$ HGG	Cultured LAK cells lysed cultured glioma cells ($n = 6$)	Slight clinical (but not radiologic) improvement.
[92]	$n = 20$ recurrent HGG	—	Median survival: 63 weeks
[93]	$n = 19$ (GBM: 5, AA:4, Other 10)	—	CR: 1, PR: 2, median survival (GBM): 15 weeks
[94]	$n = 40$ recurrent GBM	—	Median survival: 17.5 months (significantly longer than contemporary patients)
Others: [94, 143–147]			

Table 3: Selected clinical trials using cytotoxic T lymphocytes (CTLs).

Reference	Patients	Immunologic response	Clinical response
[97]	$n = 4$ (GBM: 3, AA: 1)	—	PR: 3
[98]	$n = 12$ (GBM: 6, AA: 1, Other: 5)	—	PR: 4
Others: [20, 97, 148–154]			

2.3. Antigen Identification and Targeting. Targeting of tumor-specific antigens is a promising strategy for delivering antitumor immunotherapy. The effectiveness of this approach remains controversial, however, as many vaccine trials have not demonstrated a consistent antitumor response or survival advantage despite increased tumor reactive cytotoxic T cells [102–105]. One of the challenges facing therapy directed against single antigens is the ability of a tumor to alter its antigen expression profile, resulting in immune editing. Immune editing consists of three phases: elimination, equilibrium, and escape [106]. The elimination phase is maintained by immunosurveillance of cancer cells by both the innate and adaptive immune system [107–109]. The equilibrium phase occurs when tumor cells survive the cytotoxic pressure exerted by immune cells. Finally, the escape phase results in uncontrolled tumor growth and often clinical manifestations of disease [106]. Often immune escape is preceded by mutations within cancer cells that facilitate immune evasion. For example, loss of HLA class I proteins [110, 111] and decreased response to IFN-γ [108, 112] have been described in adenomas of the lung and melanoma.

Another major challenge currently limiting antigen-targeted therapies is the inability to tailor therapy to an individual tumor's antigen expression profile. The current classification scheme for glioma does not account for the molecular diversity of GBM. A new model for classification, reported by Verhaak et al., is a molecular classification of glioblastoma consisting of four clinically relevant tumor subtypes—classical, mesenchymal, proneural, and neural [113]. A comprehensive understanding of which antigens are present on each GBM subtype would allow for better targeted immunotherapy.

2.3.1. EGFRvIII. The epidermal growth factor receptor vIII (EGFRvIII) is a truncated form of the wild-type EGF receptor [114, 115] and is an attractive antigen for immunotherapy because it is not expressed by normal brain and leads to enhanced tumorigenicity of the EGFRvIII-expressing cell [116]. This truncated protein is constitutively active despite its inability to bind extracellular ligand [117]. Efforts to target EGFRvIII, however, have been significantly hampered by immune editing [106]. For example, unpublished data from CDX-110 clinical trials reported the EGFRvIII antigen was not expressed on recurrent tumors in 20/23 patients who had been initially treated with the EGFRvIII vaccine [25].

The novel EGFRvIII epitope exists extracellulary and is a prime target for monoclonal antibody recognition [118, 119], which stimulates antitumor cytotoxic T cell maturation. EGFRvIII-specific titers are not found in normal volunteers, but are present in patients with EGFRvIII-expressing cancers, such as adenocarcinomas and gliomas [120, 121]. Early animal studies using vaccination strategies against EGFRvIII reported increased numbers of tumor-infiltrating CD4+, CD8+, natural killer (NK) cells, and macrophages as well as a dramatic increase in survival [119, 122–125].

These promising preclinical results lead to early-phase I studies looking at the use of vaccine strategies against the EGFRvIII peptide. The first study for malignant gliomas was the Vaccine for Intra-Cranial Tumors I (VICTOR1).

In this study, autologous mature dendritic cells were pulsed with 500 ug of PEPvIII, which was conjugated with keyhole limpet hemocyanin (KLH). Following surgical resection and completion of radiation therapy, all patients were vaccinated three times; the first three patients were dosed with 3×107 mature DCs per vaccine while the remaining patients were dosed with one third of their DCs per injection. No serious adverse events were reported and immunological responses were detected *ex vivo*. For patients with GBM, the median time to progression (TTP) was 46.9 weeks and median survival was 110.8 weeks. These results compare favorably with patients treated with resection site carmustine wafers [126] or temozolamide [1, 121].

The follow-up phase II study, A Complementary Trial of an Immunotherapy Against Tumor Specific EGFRvIII (ACTIVATE) evaluated the efficacy of the PEPvIII-KLH and granulocyte macrophage-colony stimulating factor (GM-CSF) [127]. Patients received three vaccinations at two-week intervals. Similar to the VICTOR1 study, there were no serious adverse effects and cellular immune responses were detected *ex vivo*. The median TTP was 14.2 months and the median survival was 32 months. Of note, upon histological examination, recurrent tumors did not express EGFRvIII.

The currently ongoing ACTIVATE II trial was initiated to evaluate the effectiveness of adding adjuvant PEPvIII-KLH vaccination therapy to standard of care (resection, temozolomide, and radiation). Of note, temozolomide induces lymphopenia, theoretically decreasing the efficacy of an immune-based therapy. Therefore, the EGFRvIII vaccine (CDX-110) was given on day 21 of the 28 day cycle, allowing recovery of the immunosuppression caused by temozolamide [128].

2.3.2. IL-13 Receptor α2. The IL-13Rα2 antigen is a promising target for immunotherapy because it is highly expressed on glioma cells but not on host CNS cells [129, 130]. However, it should be noted that IL13Rα2 expression is often heterogeneous [131]. In a study by Okano et al., it was shown that a novel epitope of IL-13Rα2 induced CD8+ T cells to secrete IFNγ and lyse IL-13Rα2-expressing glioma cells *in vitro*. This effect was only seen in CD8+ T cells expressing the *HLA-A*0201* allele [132], which 40–50% of Caucasians and Asians express [133]. To target the IL-13Rα2 in vivo, IL-13 was tagged with a mutated form of the pseudomonas exotoxin [134–138]. This fused protein (IL-13-PE38QQR), also termed Cintredekin besudotox (CB), showed promise *in vivo*; Kawakami et al. reported that CB injected intracranially resulted in both tumor regression and prolonged survival by 164% as compared with control animals [139].

Three phase I studies were undertaken to determine the safety of intracerebral administration of CB. Pooled results of the 51 total patients indicated a slight survival advantage as compared with BCNU wafers. Subsequently, 276 patients were enrolled in a Phase III study (PRECISE) to determine if the overall survival, safety, and quality of life differ in patients receiving the CB via local Convection-enhanced delivery (CED) compared to patients receiving BCNU wafers. There was no reported difference in median survival (36.4 weeks for the patients receiving CB compared with 35.3 weeks for the patients receiving Gliadel wafers, $P = 0.476$) [140, 141].

2.3.3. IL-4 Receptor. IL-4 receptor (IL-4R) is an attractive target for immunotherapy because tumor cells express a different IL-4R isoform than that which is present on circulating immune cells. This isoform of the IL-4R is commonly expressed in human gliomas and not on neural tissue [178–181]. The type 2 IL-4R signals through the Jak-STAT pathway, activating the Jak1/Jak2 tyrosine kinases, and eventually activating the STAT-6 protein, which translocates to the nucleus and regulates gene expression [182–184]. To target the IL-4R, IL-4 was fused to pseudomonas exotoxin (IL-4(38-37)-PE38KDEL) [181, 185]. Joshi et al. showed that this construct induces glioma cell death in culture [186]. *In vivo* studies demonstrated the same construct decreased the size of implanted human-derived glioma tumors (U251) with all treated mice showing complete regression. The tumors recurred in 50% of animals but were smaller than tumors harbored by control animals [187].

A phase I clinical trial of the IL-4-fused protein (cpIL4-PE) was performed in patients with recurrent malignant gliomas. The construct was injected intratumorally by CED. The authors concluded that direct glioma injection of cpIL4-PE was safe, had no systemic toxicity, and caused necrosis of malignant gliomas that were refractory to conventional therapy. Subsequent clinical trials using the same construct, with stereotactic injection as the delivery method, showed similar findings of safety and efficacy [142].

In addition to identifying appropriate epitopes, an effective immunotherapy strategy must be able to efficiently target these antigens *in vivo*. Dendritic cell, autologous tumor cell, and heat shock protein vaccines are discussed below with general principles illustrated in Figure 2.

2.3.4. Dendritic Cells. Dendritic cells (DCs) are "professional" antigen-presenting cells (APCs) that activate innate and adaptive immune responses [155]. Strategies using DCs seek to exploit this ability as GBM cells are unable to reliably present antigens to the immune system [188, 189]. DCs can be harvested from peripheral blood or bone marrow, pulsed with tumor lysate or tumor-specific peptides, and after maturation, injected back into the patient.

In a phase I trial, Yu et al. expanded peripheral blood cells *ex vivo* into DCs and pulsed them with peptides eluted from the surface of cultured autologous brain tumor cells. Seven patients received three biweekly intradermal vaccinations of peptide-pulsed DCs with no systemic side effects. The vaccination led to significant T-cell-specific cytotoxicity against glioma tumor cells and later biopsy showed that cytotoxic and memory T cells were able to traffic into the tumor [155]. Liau et al. reported a series of 12 patients treated with 1, 5, and 10 million autologous dendritic cells pulsed with autologous tumor peptides. Similar to the previous studies, no systemic side effects were seen and survival was improved compared to historical controls. Of note, the magnitude of the T cell infiltration was inversely correlated with TGF-β expression within the tumor microenvironment [156].

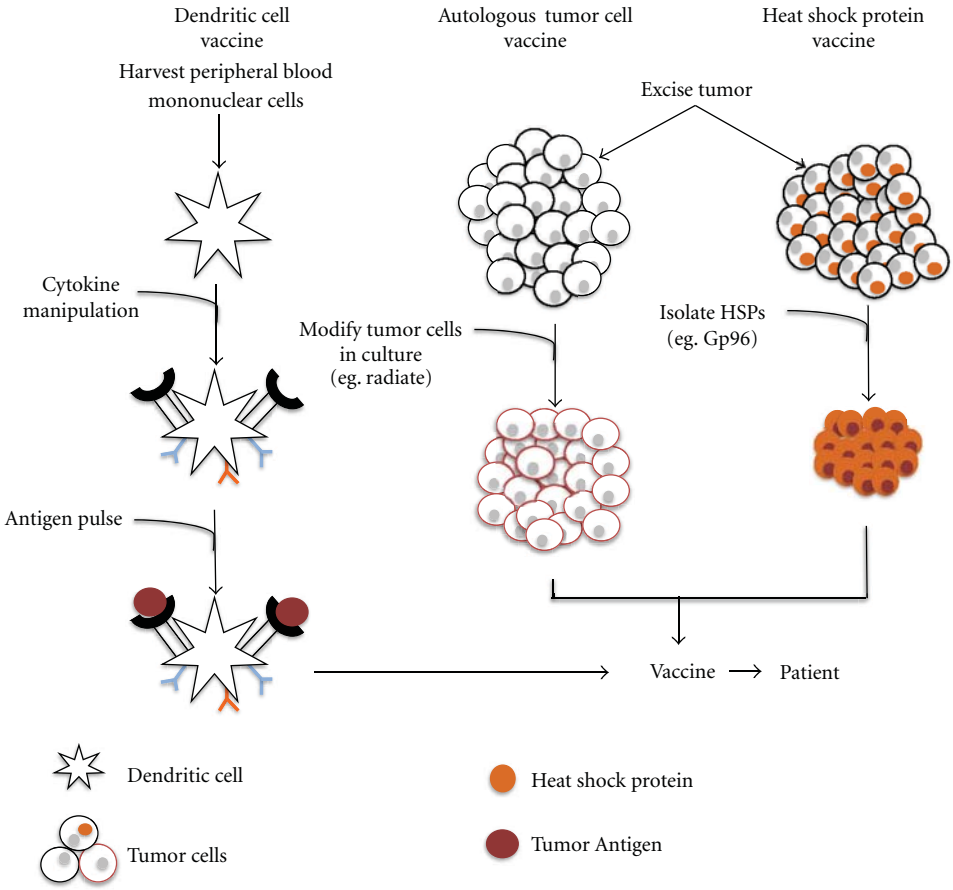

Figure 2: Vaccine Strategies for GBM. (From Left) *Dendritic cell vaccine:* peripheral blood mononuclear cells are isolated from the patient and cultured *ex vivo*. Cytokines are added to culture to activate the DCs. The matured DCs are pulsed with tumor antigen and then added to the vaccine preparation. *Autologous tumor cell vaccine:* after tumor removal, tumor cells are cultured. In some cases, these cells are modified (e.g., radiation, chemical) and then injected back into the patient. *Heat shock protein vaccine:* after tumor removal, tumor cells are cultured and specific heat shock proteins (e.g., Gp96) are isolated and purified. The proteins are then added to the vaccine preparation and injected into the patient.

A larger trial showed 8 of 19 patients with GBM had a median survival of 33.6 months with a median time to progression of 18.1 months, surpassing that of historical controls receiving standard of care. Of note, 42% of patients have survived longer than 4 years [190].

Pulsing DCs with whole tumor lysate increases the number of targeted epitopes and prevent antigen-loss escape and immune editing [191]. Parajuli et al. reported that DCs pulsed with apoptotic tumor cells or total tumor RNA led to a more robust immune response compared to DCs pulsed with tumor cells or fused with glioma cells [192]. Clinical trials using dendritic cells are summarized in Table 4.

2.3.5. Autologous Tumor Cells. The use of autologous tumor cells (ATCs) as an immunotherapeutic approach has garnered attention due to the ability to activate the immune system with an increased number of potential glioma antigens. Several strategies for ATC vaccines have been tested including using irradiated glioma cells that were either autologous or allogenic. The autologous strategy was more beneficial in providing the most relevant antigens to the patient's tumor [193–195]. Recent clinical trials have shown this method can be used without systemic side effects. Schneider et al. reported 11 patients who received an autologous tumor vaccine with cells modified with Newcastle-Disease-Virus after surgery and radiation. Survival was no different compared to patients receiving surgery, radiation, and chemotherapy. No side effects were seen with the vaccine group [174]. A similar trial by Steiner et al. reported 23 patients who underwent surgery, radiation, and vaccination. There was a statistically significant increase in median progression-free survival (40 weeks versus 26 weeks in controls) and median overall survival of vaccinated patients (100 weeks versus 49 weeks in controls) [175]. Using an autologous formalin-fixed tumor vaccine, which is thought to preserve the antigenicity of the tumor cells, Ishikawa et al. studied 24 patients who received surgery, and radiation, showing no adverse events [176]. Selected clinical trials using ATCs are summarized in Table 5.

2.3.6. Heat Shock Proteins. Heat shock proteins (HSPs) are chaperon proteins that aid in protein folding and are

Table 4: Selected clinical trials using dendritic cells (DCs).

Reference	Patients	Immunologic response	Clinical response
[155]	$n = 7$ (GBM: 6, AA: 1)	Cytotixic and memory T cells found in recurrent tumor bulk	Median survival: 455 days (Control group: 257 days)
[156]	$n = 12$ (GBM: 7, Recurrent GBM: 5)	Cytotoxicity against autologous tumor cells. Cytotoxic T cells found in recurrent tumor bulk.	Median TTP: 19.9 mo ($P = 0.028$), Median survival: 35.8 mo ($P = 0.006$)
[157]	$n = 18$ EGFRvIII expressing GBM	82% of recurrent tumors lost EGFRvIII expression	Median survival: 26 mo ($P = 0.001$)
Others: [80, 84, 158–173]			

Table 5: Selected clinical trials using autologous tumor cells (ATCs).

Reference	Patients	Immunologic response	Clinical response
[174]	$n = 11$ recurrent GBM	Local skin reaction	Median survival: 46 weeks
[175]	$n = 23$ GBM	Delayed-type hypersensitivity, increased memory T cells, increased CD8+ T cells in recurrent tumors	Median progression free survival: 40 weeks, median survival 100 weeks
[176]	$n = 12$ GBM	—	CR: 1, PR: 1, minor response: 2, median survival: 10.7 mo

Table 6: Selected clinical trials using heat shock proteins (HSP).

Reference	Patients	Immunologic response	Clinical response
[177]	$n = 12$ recurrent GBM	—	Median survival: 10.5 mo

implicated in mediating adaptive and innate immune responses [195]. While there are five major families of HSPs, the HSPs Grp 96, HSP 90, HSP 70, HSP 110, and HSP 170 are considered the most immunogenic [196, 197]. HSPs aid in the folding of many proteins within the cell, and, therefore, a specific target antigen is not required, thus decreasing the potential for immune editing. Furthermore, HSPs have been shown to induce human DC maturation and to activate DCs to secrete proinflammatory cytokines making this strategy an attractive option for immunotherapy.

Clinical trials using a vaccine-based HSP strategy are currently underway. In cancers, such as metastatic melanoma, colorectal carcinoma, chronic myeloid leukemia, and renal cell carcinoma, HSP vaccines have been shown to be safe and associated with increased survival [198–201]. Parsa et al. reported a study in 12 patients with recurrent GBM, seven of the eight patients treated had a median survival time of 10.5 months compared to historical controls' median of 6.5 months [177]. Currently, two phase I/II clinical trials using the Grp 96 vaccine strategy are underway (NCT00293423, NCT00905060). Selected clinical trials using HSPs are summarized in Table 6.

3. Challenges in the Tumor Microenvironment

3.1. Cell Populations. GBM-mediated immunosuppresion arises from coordinated interactions among the diverse cell populations, cytokines, and extracellular matrix proteins in the tumor microenvironment. The nature of these interactions is yet to be fully characterized, but is likely to be more complex than initially appreciated. For example, it has been shown that 20–90% of endothelial cells in GBM-associated vasculature harbor the same mutations as the tumor cells [202] and that a subpopulation of CD133+ tumor stem cells expresses vascular-endothelial cadherin (CD144) [203]. Taken together, these findings indicate that a significant number of GBM-associated endothelial cells may arise from tumor stem cells [204]. In addition, experiences with conventional therapies have highlighted how specific cell populations give rise to resistance. For example, tumor stem cells are largely radioresistant. A recent study by Tamura et al. found that tumors in a cohort of patients with recurrent grade III and IV gliomas following treatment with radiosurgery and external beam radiation therapy were significantly enriched for CD133+ cells [205]. Interestingly, additional cell populations have been implicated in this phenomenon as well. *In vitro* studies of GBM stem cell sensitivity have not clearly demonstrated that these cells are more radioresistant than CD133− tumor cells [206]. Based on these findings, Calabrese et al. have proposed that the resistance of glioma stem cells to radiotherapy may arise from interactions within the GBM microenvironment [207]. Supporting this theory is the observation that GBM stem cells tend to reside within perivascular niches, where interactions with endothelial cells appear to impart

tumor stem cell radioresistance [204, 208]. Other lines of evidence indicate that extracellular matrix proteins and hypoxia within the tumor microenvironment may impart radioresistance in tumor stem cells. These two examples illustrate the fact that an effective immunotherapy must not only target tumor cells, but must also disrupt the immunosuppressive activities of a variety of cell populations in the tumor microenvironment.

3.2. Cytokines. GBM cell lines have long been known to express high levels of immunosuppressive cytokines [209]. However, our understanding of the origins of these cytokines and the roles they play in the tumor microenvironment represents one of the most significant challenges to cytokine-based therapies for GBM. A recent study by Rodriques et al. demonstrated that expression of IL-10, TGF-β, and B7-H1 is induced in normal human monocytes after exposure to GBM cells [37]. TGF-β has also been implicated in the transformation of vascular endothelial cells to a proangiogenic phenotype characteristically associated with GBM [46]. Other studies indicate that TGF-β and IL-10 are more highly expressed in CD133+ than in CD133− glioma cells and that elevated expression of these cytokines specifically within tumor stem cell population correlates with a poorer prognosis [45, 210]. In order to fully understand the relationship between specific cytokines and the variety of cell populations present in the GBM microenvironment, subclassification of these cell populations may be necessary. For example, it has been suggested that the level of TGF-β expression as well as the effects of TGF-β signaling may vary among cancer stem cell subtypes [211]. Another recent study has shown that exposing GBM cells to IFN-γ decreased TGF-β but increased expression of PD-1 ligand and Indoleamine-2,3-Dioxygenase (IDO) [212]. It is reasonable to speculate that other immunosuppressive cytokines exhibit comparably complex interactions.

4. Therapies Directed at the Immune Microenvironment

4.1. STAT3 Blockade. STAT3 is a member of the signal transducer and activator of transcription (STAT) family of transcription factors. The detailed activities of STAT3 in cancer are reviewed elsewhere [213]. In brief, STAT3 is activated when Janus kinases (JAKs) phosphorylate the cytoplasmic tail of activated IL-6 family cytokine receptors [214]. The phosphorylated receptor then recruits STAT1 and STAT3 via the Src homology 2 (SH2) domain of the STAT protein [214, 215]. JAK tyrosine kinase activity subsequently phosphorylates STAT3 on Tyr 705, leading to formation of a phosphorylated-STAT3 (p-STAT3) homodimer which translocates to the nucleus and binds several promoters which regulate cytokine expression, cell differentiation, proliferation, apoptosis, and angiogenesis [216–219]. Constitutive activation of STAT3 has been implicated in the tumorigenesis of many cancers both inside and outside of the CNS and has been shown to be sufficient to transform cells to a malignant phenotype *in vitro* [220].

Some authors have reported that p-STAT3 is present in high levels in GBM cell lines [221] and in greater than 75% of tumor tissue samples [222]; however, other authors have failed to corroborate these findings [27]. In tumors exhibiting high levels of STAT3 activity, this transcription factor has emerged as a critical convergence point for many pathways known to be associated with GBM growth and invasion. In addition, increased STAT3 activation has been correlated with shorter overall survival in a cohort of patients with GBM [222].

Numerous lines of evidence indicate a protumorigenic role for STAT3 in the GBM microenvironment. STAT3 activation has been shown to be increased in GBM under hypoxic conditions, leading to elevated expression of proangiogenic factors such as vascular endothelial growth factor (VEGF) and hypoxic inducible factor-1 (HIF-1) [223]. Furthermore, STAT3 inhibition results in a reduction in endothelial cell tube formation *in vitro* [216, 223]. STAT3 has also been implicated in tumor invasion and suppression of apoptosis. For example, Chen et al. recently demonstrated that STAT3 inhibition reduces expression of the proinvasive factor matrix metalloprotease-2 (MMP-2) and the antiapoptotic factors Bcl-xL and survivin [224]. STAT3 is also critical for maintaining tumor stem cells [225]. A recent study by Villalva et al. demonstrated that siRNA knockdown or inhibition of STAT3 with the small molecule inhibitor Stattic led to decreased GBM stem cell proliferation and inhibited neurosphere formation [226]. In addition to its roles in angiogenesis, tumor invasion, apoptosis, and maintenance of tumor stem cells, STAT3 is known to act as a potent inhibitor of both innate [227] and adaptive [228] immune responses. STAT3 also induces tolerance via Treg activity, potentially through an HIF-1-mediated mechanism [229].

Although STAT3 has been most extensively studied as a tumor-promoting factor in GBM, evidence has recently emerged to suggest that it may act alternately as a protumorigenic factor or a tumor suppressor based on the genetic background of the tumor [230]. The theory that STAT3 may exert tumor-suppressing effects in GBM originated from the observation that STAT3 plays a prominent role in astrocyte differentiation [231, 232]. Studies of STAT3−/− astrocytes have demonstrated that these cells exhibit increased proliferation and invasion, although this mutation is not sufficient to produce malignancy [230]. In addition, STAT3 suppresses malignant transformation of astrocytes deficient in PTEN in an orthotopic transplant model in SCID mice [230] and a correlation between PTEN mutation and low levels of STAT3 activity has also been reported in human GBMs [233]. Conversely, STAT3 appears to be protumorigenic in EGFRvIII-expressing tumors [230]. The details of STAT3's interaction with EGFRvIII are currently unknown; however, evidence from breast cancer cell lines suggests that EGFRvIII may translocate to the nucleus and alter the binding of STAT3 to DNA [234].

The multiplicity of pro-oncogenic effects ascribed to STAT3 makes this transcription factor an attractive target for immunotherapy. Strategies to block STAT3 in GBM have focused primarily on direct inhibition using RNA interference and small molecule inhibitors or indirect inhibition

by targeting upstream kinases or regulatory SOCS proteins [221, 235–237]. Although STAT3 inhibition has yielded promising results *in vitro*, applying this approach to animal models of GBM has produced mixed outcomes. In light of the finding that STAT3 may be alternately protumorigenic or suppressive to tumor growth, additional research is needed to elucidate the role of STAT3 in a variety of genetic contexts, including the background genotype of the host.

Even if the correct patients are identified, the tumor microenvironment may pose a number of additional challenges to effective GBM therapy with STAT3 blockade. For example, although inhibiting STAT3 may overcome some of the immunosuppressive mechanisms employed by GBM, immune cells must still efficiently identify appropriate tumor-specific antigens in order to avoid immune editing. In addition, evidence has already emerged to suggest that cancer stem cells express a different immunosuppressive cytokine profile in response to STAT3 blockade than bulk tumor cells [238]. This finding highlights the principle that it will be critical to consider the effects of STAT3 inhibition on cytokine expression and signaling in the variety of cell populations present in the GBM microenvironment individually as well as in aggregate. Even if STAT3 inhibition results in generation of an antitumor immune response, this activity may be thwarted by activation of immune checkpoints such as PD-1 [29] and CTLA-4 [26]. Other barriers to STAT3 inhibition in the treatment of brain tumors include identifying small molecule inhibitors that can either cross the blood-brain barrier or be delivered locally. Nevertheless, STAT3 remains one of the most promising targets in immunotherapy for GBM and at least one small molecule inhibitor, WP1066, is currently in preclinical development.

4.2. Regulatory T Cell Depletion. Tregs are a CD25+, FoxP3+ subset of CD4+ helper T cells which suppress immune activation through interactions with T cells, B cells, NK cells, DCs, and macrophages [239–243]. Tregs have been shown to express CTLA-4, to decrease the secretion of IL-2 and IFN-γ [244], and to skew the immune response away from a cytotoxic Th1-mediated response in favor of a Th2 response [245]. Studies of human GBM tissue samples have reported tumor-infiltrating lymphocyte populations significantly enriched for Tregs [26]. GBM cells also appear to secrete high levels of CCL22 and CCL2, which facilitates Treg trafficking to the tumor [246]. In addition, high-grade gliomas have been reported to exhibit a higher density of Tregs than low-grade tumors [247]. These observations have led to interest in developing immunotherapies for GBM that target Tregs.

Tregs have been shown to be associated with a number of other known immunomodulatory pathways [248]. For example, the STAT3 inhibitor WP1066 has been shown to decrease Treg proliferation. In addition, CTLA-4 blockade may abrogate the immunosuppressive effects of Tregs in the tumor microenvironment without directly inhibiting their immunosuppressive properties [249–251]. Direct inhibition of Tregs is also possible with anti-CD25 antibodies and has been shown to improve survival in mouse glioma models [252]. A number of other approaches have also been proposed to inhibit Tregs in gliomas. These approaches are reviewed in detail elsewhere [253].

Indirect evidence for the efficacy of Treg depletion in human glioma comes from combining immunotherapy with cyclophosphamide, which preferentially inhibits Treg activity at low doses [254]. Clinical trials combining cyclophosphamide with a dendritic cell vaccine for renal cell carcinoma [255] or with a protein antigen vaccine for breast cancer [256] have demonstrated that the addition of cyclophosphamide augmented the antitumor effect. Blocking antibodies against CTLA-4 [249] and CD25 [252] have been shown to be effective against gliomas in mice; however, neither of these approaches has been evaluated in clinical trials.

One of the primary challenges impeding the development and implementation of Treg depletion for treatment of GBM is precisely delineating how these cells interact with the other immunosuppressive factors in the tumor environment. Despite numerous lines of evidence implicating a protumorigenic role for Tregs, and the theoretical appeal of these cells as targets for immunotherapy, fundamental questions about the role of Tregs in GBM tumorigenesis remain unanswered. For example, several studies have failed to convincingly correlate the density of tumor-infiltrating lymphocytes with prognosis in human gliomas [257–259]. Because these studies did not account for lymphocyte activity, it has been proposed that local immunosuppression in GBMs results from inhibition of T cell function secondary to an enriched population of Tregs [247]. Studies directly evaluating the relationship between Treg fractions and survival in patients with GBM, however, have not demonstrated a reliable correlation [260].

Tregs have been implicated in association with many other known immunosuppressive factors in the GBM microenvironment, such as CTLA-4 and STAT3. The lack of a clearly defined mechanism underlying the interactions between Tregs and CTLA-4, however, precludes the development of maximally effective combination therapies. The finding that STAT3 blockade inhibits Treg function is intriguing and deserves further exploration. In particular, STAT3 signaling may coordinate the activities of Tregs with other cell populations in the tumor microenvironment, including tumor stem cells [238]. Ultimately, defining the roles of Tregs in GBM represents a critical step toward understanding the mechanisms underlying the immunosuppressive tumor microenvironment and may serve as a valuable target for intervention.

5. Conclusion

We have reviewed challenges presented by the tumor microenvironment and many of the current approaches to immunotherapy for GBM. It is becoming increasingly clear that GBM-mediated immunosuppression arises not only from the intrinsic properties of tumor cells, but from the ability of these cells to coordinate the activities of a diverse set of cell types and signaling pathways in the tumor micro-environment. Therefore, the development of effective immunotherapies will require careful study of how intervening at any point in this system alters the dynamics of

these interactions. For example, the finding that treatment with IFN-γ increases expression of PD-L1 demonstrates potentially redundant immunosuppressive mechanisms. The differential effects of STAT3 blockade based on tumor genetics highlights the importance of developing molecular classification schemes that reflect responsiveness to various immunotherapy approaches. Furthermore, the finding that tumor stem cells may differentiate into vascular endothelial cells suggests potential interactions between tumor endothelial cells and immune cells that have not yet been elucidated. With these challenges, however, comes enormous potential to precisely target the defense mechanisms in GBM and tip the balance back in favor of the immune system.

Abbreviations

CR: Complete response
PR: Partial response
SD: Stable disease
TTP: Time to progression
GBM: Glioblastoma multiforme
AA: Anaplastic astrocytoma.

Authors' Contribution

C. Jackson and J. Ruzevick contributed equally to this work.

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Review Article

From Tumor Immunosuppression to Eradication: Targeting Homing and Activity of Immune Effector Cells to Tumors

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Unraveling the mechanisms used by the immune system to fight cancer development is one of the most ambitious undertakings in immunology. Detailed knowledge regarding the mechanisms of induction of tolerance and immunosuppression within the tumor microenvironment will contribute to the development of highly effective tumor eradication strategies. Research within the last few decades has shed more light on the matter. This paper aims to give an overview on the current knowledge of the main tolerance and immunosuppression mechanisms elicited within the tumor microenvironment, with the focus on development of effective immunotherapeutic strategies to improve homing and activity of immune effector cells to tumors.

1. General Introduction

In the beginning of the 20th century, the concept according to which the immune system can be manipulated for tumor prevention or tumor treatment has emerged. Around half a century later, Burnet postulated the existence of a complex immunological mechanism capable of eliminating potentially malignant cells and, thus, gave birth to what would afterwards be called the cancer immunosurveillance theory [1]. In later years, strong evidence supporting the existence of intricate antitumor immune responses lead to the more exhaustive concept of cancer immunoediting. According to this concept, the multistep process of cancer development consists of three phases. The first phase, of elimination, is similar to the cancer immunosurveillance theory. Malignant cells, generated after genetic modifications that may occur during cell division cycles, present the singular property of expressing tumor antigens, a feature which makes them immunologically distinguishable from nonmalignant cells. Recognition of these tumor antigens by cells belonging to the host immune system leads to development of antitumor immune responses. Within the second phase, of equilibrium, a dynamic balance between the tumor microenvironment and the host immune responses is established. However, due to the negative activity of the tumor microenvironment as a dynamic inducer of immune cell anergy or death [2, 3], these antitumor immune responses are apparently insufficient to completely eradicate tumors. Hence, the third phase, of escape, consists of development of immune resistant tumor variants into fully grown and progressive clinical tumors [4, 5]. Here, the concept of cancer immunotherapy comes into play. Although the host immune system is clearly capable of recognizing cancer cells [6], the ability to which it can control tumor growth remains very limited. Different explanations can be envisaged to justify the decreased antitumor activity of the immune system. All of them take into account two major obstacles: on one hand, reduced homing of immune cells to the tumor site and, on the other, hampering of the antitumor immune functions due to tumor microenvironment or immunomodulatory properties of suppressive cell populations. Cancer-directed immunotherapies encompass diverse attempts either to stimulate the antitumor immune system or to inactivate and deplete protumor immune cell populations. Effective antitumor immunotherapeutic strategies take into account the complex interplay between innate, nonspecific and adaptive, antigen-specific, immune responses.

This paper aims to give an overview on the current knowledge of the main tolerance and immunosuppression mechanisms elicited within the tumor microenvironment, with the focus on development of effective immunotherapeutic strategies to improve homing and activity of immune effector cells to tumors.

2. The Balance of Immune Surveillance in the Tumor: Navigating between Scylla and Charybdis

An increasing body of evidence substantiates the concept that specific cell populations from both the innate and adaptive immune systems interact with developing tumors and frequently contribute to the arrest of tumor growth and induce tumor regression in animal models and cancer patients. To counteract the antitumor activity of these effector cells, regulatory cell populations have emerged, capable of suppressing the antitumor immune responses through a large array of mechanisms. These silencing or suppression mechanisms can be functionally divided in two main categories: tolerance mechanisms, characterized by the absence of an immune response only to a specific set of antigens and maintenance of normal responses to all other antigens and immunosuppression mechanisms, characterized by an impaired ability of the immune system to fight cancer development.

2.1. Induction of Tolerance Mechanisms. Most often, tolerance mechanisms are directed against the antitumor activity elicited by cell populations belonging to the adaptive immune system. The main targets of these tolerance mechanisms are Th1 $CD4^+$ T cells and cytotoxic $CD8^+$ T lymphocytes (CTLs). Apart from these adaptive immune populations, dendritic cells (DCs) are a distinct cell subset with the capacity to initiate primary and secondary T-lymphocyte responses against developing cancer, thus representing a putative target for tolerance induction. Both the importance and relevance of these immune populations and the tolerance mechanisms they are the target of are shortly addressed below.

2.1.1. Dendritic Cells. Alongside macrophages and B lymphocytes, DCs comprise one of the three main professional APC populations. Within the context of tumor development, their crucial importance stems from the capacity to engulf, process, and present tumor-associated antigens (TAAs) and thus generate tumor-specific immunity. Generation of potent antitumor immunity by DCs is the result of a complex process comprising three major steps: proper presentation of TAAs to T lymphocytes, activation of T lymphocytes in a specific manner as a response to TAAs presentation, and homing of these specific T cells to the tumor site, where they exert cytolytic activity against tumor cells expressing the TAAs [7]. Tumor escape mechanisms developed during cancer progression can occur at any of these various levels. With respect to the first step, these escape mechanisms generally translate into a deficit in antigen presentation. This deficit stems from two major sources: on one hand, a decreased number and function of APCs, and on the other, a semimature phenotype. One of the earlier studies indicating the effects of defective antigen, presentation by DCs is performed in a murine model bearing tumors transfected with a human p53 minigene. Both *in vitro* restimulation of T cells isolated from either control or tumor-bearing mice and *in vivo* induction of CTLs by DCs from tumor-bearing mice were significantly decreased in comparison with the same effects exerted by DCs isolated from control mice [8]. Later research in this direction further substantiates these findings in various clinical models. A study performed on DCs isolated from renal cell carcinoma patients indicates that less than 10% of the total DC population represents activated cells capable of antigen presentation and T cell stimulation [9]. The situation proves to be similar in patients with both advanced breast cancer [10, 11] and non-Hodgkin's lymphoma [12]. Moreover, DCs exposed to indoleamine 2,3-dioxygenase [13], transforming growth factor-beta (TGF-β) or prostaglandins, have been shown to induce tolerance and anergy leading to failure of recognizing tumor cells.

2.1.2. Th1 $CD4^+$ T lymphocytes. Although not directly capable of antitumor activity due to their lack of cytotoxic and phagocytic properties, $CD4^+$ T cells, also known as mature T helper cells, play a crucial role in the initiation and activation of the antitumor immune response. In accordance with their phenotypic characteristics and function, $CD4^+$ T cells can be divided in two types. Type 1, IL-12 polarized $CD4^+$ T cells (Th1) provide help to cytotoxic $CD8^+$ T cells, amongst others by stimulating their proliferation and inducing IFN-γ secretion once antigen-specific immunity has developed [14]. In contrast, type 2, IL-4 polarized [15] $CD4^+$ T cells (Th2) secrete cytokines which induce neutralizing antibody production by B cells, thus directing immunity towards a tumor-promoting type 2 response.

2.1.3. Cytotoxic $CD8^+$ T lymphocytes. Cytotoxic T cells constitute a subgroup of T lymphocytes able to induce death of tumor cells and infected or otherwise dysfunctional somatic cells, following their activation. The activation process of cytotoxic $CD8^+$ T lymphocytes relies on various simultaneous interactions between molecules which are expressed on the surface of the $CD8^+$ T cell itself and corresponding molecules located on the surface of the antigen-presenting cell (APC). The first activation cue of $CD8^+$ T cells consists of the interaction between their membrane T cell receptor (TCR) and peptide-bound MHC class I molecules located on the surface of APCs. Following this cue, a second signal comprising of interactions between the costimulatory molecules CD28 (located on the surface of $CD8^+$ T cells) and CD80 or CD86 (located on the surface of APCs) can develop. Depending on the case, this second signal can be substituted by cytotoxic T cell stimulation with cytokines released by helper T cells. Similarly to $CD4^+$ T cells, $CD8^+$ T cells can also be divided in different subsets [16], according to their phenotypical and functional properties. Naïve $CD44^{low}$ $CD8^+$ T cells are differentiated mature T

cells that have not yet encountered their cognate antigen in the periphery. Upon antigen recognition, they become memory $CD44^{high}$ $CD8^+$ T cells with a higher sensitivity to TCR/CD8 signaling in response to subsequent antigen stimulation [17]. Effector memory $CD8^+CD44^+$ T cells (Tem), characterized by low expression of markers necessary for cellular extravasation (e.g., CD62L), have been shown to restore systemic antitumor immunity in mouse models of lung and mammary carcinoma [18]. When compared with Tem cells, central memory T cells (Tcm), phenotypically defined as $CD8^+CD44^+CD62L^+CD127^+$, confer superior immunological protection against viruses, bacteria [19], and cancer [20]. antitumor effector T cells can be obtained by systemic delivery of IL-12 and GM-CSF to tumors or by activation of tumor-resident $CD8^+$ T effector/memory cells [21]. By releasing various cytokines, such as perforin and granzyme B, these effector T cells are capable of inducing apoptotic death of tumor cells. The activity of these adaptive immune-cell populations is being continuously targeted by the tumor microenvironment, through a versatile array of either tolerance or immunosuppression mechanisms.

2.1.4. Tolerance Mechanisms. When talking about cancer development and progression, one should take into account two main types of alterations within the tumor environment: effector-cell related tolerance or immunosuppression and tumor-cell associated alterations. Intrinsic alterations of the tumor cells lead to decrease or disappearance of immunogenicity, whereas extrinsic alterations are induced by the tumor cells themselves, however exerting their activity on effector T cells within the tumor microenvironment. The latter of the two comprises more varied and versatile escape mechanisms, as they can either elicit a proximal effect, on the surrounding microenvironment or a distant effect, on the host immune system giving rise to the state of immunological tolerance. A proximal effect of colon cancer cells, which leads to evasion of FasL mediated cell death, is secretion of decoy receptors that bind and neutralize FasL [22]. On the other hand, distant effects exerted on the host immune system consist of a wide array of tolerance mechanisms. One very efficacious tolerance mechanism is deletion of effector T cells due to expression of death-inducing ligands by cancer cells [23, 24]. Direct tolerization of antitumor T cells by tumor cell-induced TGF-β signaling is another highly effective mechanism, leading to inhibition of master transcriptional regulators of $CD4^+$ T cells [25] and significantly decreased function and frequency of CTLs (cytotoxic T lymphocytes) in a thymoma mouse model [26]. The main tolerance mechanisms leading to decreased numbers of antitumor effector T cells, coupled with increased numbers of low-affinity autoreactive T cells [27], are constituted by ignorance and anergy. Immunological ignorance is characterized by lack of contact with the antigens able to induce phenotypical changes, whereas anergy arises after negative regulation induced by different types of host factors (e.g., suppressor cells, their secreted cytokines) [28]. Other competent tolerance mechanisms are deficient priming of antitumor effector T cells [29] and increased expression of inhibitors which block complement mediated lysis of tumor cells [30]. However, regardless of the tolerance mechanism exerted by tumor cells, the end result consists of reduced or completely suppressed cytolytic activity of intratumoral effector T cells. Strategies aimed at increasing the activity of these immune effector cells at the tumor site will be addressed in Section 3.2 of this paper.

2.2. Induction of Immunosuppression Mechanisms. When compared to mechanisms of tolerance induction, the machinery of antitumor Immunosuppression is more versatile, since it encompasses a large variety of tools used by the tumor environment to target various mechanisms of inhibition of tumor growth and development. From a cellular point of view, the most widely encountered suppressive cell populations within the tumor environment are macrophages, myeloid derived suppressor cells, and regulatory T cells. The mechanisms by which these cell populations manage to give rise to tumor-immune escape are described below.

2.2.1. Tumor Associated Macrophages (TAMs). Tumor-induced recruitment and expansion of regulatory cell populations is aimed at both the innate and adaptive immune systems. Concerning recruitment of suppressive innate immune populations, one clear example is given by TAMs. Similarly to $CD4^+$ T cells in adaptive immunity, the innate immune populations of macrophages can be either anti- or protumorigenic, depending on their phenotype [31]. Antitumorigenic infiltrating macrophages, "classically activated" by the action of microenvironmental signals such as IFN-γ and bacterial factors, are polarized towards the M1 phenotype [32] and elicit cytotoxic activity against tumor cells *in vivo* [33], through their production of Th1 cytokines and iNOS. These macrophages also have the capacity to function as antigen presenting cells [34] that activate CTLs. On the other hand, TAMs are "alternatively" activated by Th2 cytokines such as IL-4 or IL-13 [35] towards an M2 non-cytotoxic phenotype. These M2 macrophages are frequently found in solid tumors, where they promote remodeling of the extracellular matrix and secrete growth factors, therefore, inducing tumor-specific neoangiogenesis [36]. Also, different studies have underlined their capacity to cause tumor growth both directly, by production of cytokines that stimulate proliferation of tumor cells [37], and indirectly by stimulating proliferation of endothelial cells [38]. For example, in the HPV16 E6- and E7-expressing TC-1 tumor mouse model, TAMs were shown to cause suppression of the antitumor T-cell response [39], while their secreted IL-10 cytokine subsequently induced a regulatory T cell phenotype [40].

2.2.2. Myeloid-Derived Suppressor Cells (MDSCs). MDSCs represent a highly heterogenic population of incompletely matured granulocytes, macrophages, and dendritic cells [41], with different morphology, functions, and differentiation conditions, when compared to TAMs [42]. Although MDSCs

are capable of immune response regulation in healthy individuals, it has been observed that they dramatically expand during cancer development and treatment (Draghiciu O, Walczak M, Nijman HW, and Daemen T, unpublished observations), inflammation conditions or chronic infections [43, 44]. Characterized by a high phenotypical variety, they can be generally identified in mice as $CD11b^+Gr1^+$ cells [45]. After tumor-induced expansion, they can be divided in two main subsets, depending primarily on their ancestors, but also on the suppression mechanisms they exert: monocytic MDSCs, with a $CD11b^+LY6G^-LY6C^{high}$ phenotype, and granulocytic MDSCs, with a $CD11b^+LY6G^+LY6C^{low}$ phenotype. In humans, MDSCs are characterized as $CD11b^+CD14^-CD33^+$ cells [46] and have been found to be elevated in patients with different types of cancers [42, 47, 48]. As indicated by their heterogenic composition, MDSCs can inactivate both $CD4^+$ and $CD8^+$ T cells [49–52] and therefore display a large array of mechanisms of T cell function suppression. One such mechanism is represented by tumor-induced overexpression of CD80 (B7-1) on the surface of MDSCs, to which the inhibitory CTLA-4 (CD152) molecule expressed on $CD4^+CD25^+$ T cells binds with high affinity. Binding of CTLA-4 to CD80 was shown to induce suppression of antigen-specific immune responses [53]. High production of arginase [54, 55] constitutes a common suppressive mechanism for all subsets of MDSCs. Granulocytic MDSCs particularly produce high levels of ROS [45], through signaling via the STAT3 pathway, thus leading to direct damage of the T cell DNA. In contrast, monocytic MDSCs present increased iNOS activity leading to high NO production [56]. In their turn, increased levels of NO have the capacity to induce T cell function suppression via different inhibition mechanisms of MHC class II expression [57] or STAT5 signaling cascade [58].

Other mechanisms of MDSC-induced suppression of effector T cells comprise induction of Tregs by IL-10 secretion in mouse models of colon carcinoma, B16 melanoma, and in patients with HPV-induced neoplasia [59]; depletion of cysteine, the essential aminoacid necessary for T cell activation [60]; secretion of high peroxynitrite levels, which lead to tumor progression [61] upregulation of Cox2/PGE2 [62]. However, the suppressive capacity of MDSCs has been recently questioned by a highly controversial study [63] proving that MDSCs from ascites of ovarian cancer bearing mice were immunostimulatory (they increased CTLs proliferation via CD80 signaling) and adoptive transfer of these MDSCs induced tumor regression. Lastly, immature dendritic cells (iDCs) suppress antitumor immunity by induction of Tregs [64], which in their turn inhibit HPV-specific immunity in patients with (pre)malignant cervical neoplasia [65].

2.2.3. Regulatory T Cells (Tregs). In terms of adaptive immunity, one of the most studied immunosuppressive cell populations is represented by $CD4^+CD25^+FoxP3^+$ Tregs [66–68]. Based on their phenotype and localization, Tregs can be divided into several categories: naturally occurring $FoxP3^+$ Tregs, generated in the thymus [69–72], and antigen-induced Tregs, generated in the periphery [69]. One of the main definitory characteristics of $CD4^+CD25^+$ Tregs of normal naïve mice is represented by the high expression of the TNF-receptor superfamily member GITR (glucocorticoid-induced TNFR-related protein) [73]. A more detailed subphenotypic classification of Tregs can be found in the review of Feuerer et al. [74]. Tregs can suppress the antitumor immune responses through their high surface expression of CTLA-4, the main T-cell inhibitory signal [75] which mediates attenuation of intercellular association. Moreover, $FoxP3^+$ naturally occurring Tregs (nTregs) are well-known negative regulators of antitumor immunity through different mediators, such as FoxP3 [76]. Intratumoral accumulation of FoxP3 leads to poor prognosis of gastric [77] and ovarian [78] carcinomas. Another mediator of the antitumor effects of nTregs is IL-2, needed for *in vivo/in vitro* functional Treg activation [79] and maintenance of their CD25 expression [80]. After IL-2 and TGF-β stimulation [81], antigen-induced $FoxP3^+$ Tregs [82] have also been shown to present suppressive activity.

Th17 T cells represent a proinflammatory subset of helper T cells, particularly characterized by their capacity to secrete IL-17 *ex vivo* and to constitutively express the lineage-specific factor RORyt [83]. Recent studies indicate towards a close relationship between these Th17 T cells and a distinct subset of suppressive human memory $CD4^+FoxP3^+$ Tregs [84]. IL23-induced Th17 cells [85] produce IL17, a cytokine that enhances inflammation by stimulating the expression of other pro-inflammatory cytokines and acute phase proteins. Although there are some studies which indicate an antitumoral function of Th17 cells [86], *in vitro* experiments establishing the pro- or antitumor role of these cells are equivocal. Also, secretion of IL-17 by Th17 T cells promotes neovascularization and tumor growth in a mouse model of ovarian cancer and in patients with advanced cancer [87]. Therefore, additional studies are necessary to clarify the functions of Th17 cells with regards to tumor immunity.

2.3. Other Mechanisms of Tumor Progression. The vast majority of mechanisms of tumor Immunosuppression are generated by a complex interplay of activities and factors belonging to effector-extrinsic suppressor cell populations. One such effector-extrinsic mechanism that has been shown to contribute to tumor progression involves the overexpression of some G-protein-coupled receptors (GPCRs) on the surface of endothelial cells. In some cases, the effect of this overexpression was correlated with tumor progression and metastasis.

In contrast to effector-extrinsic mechanisms of tumor development, effector-intrinsic mechanisms evolve based on the upregulation of coinhibitory receptors able to induce direct lymphocyte inactivation. Both the roles of these GPCRs and those of upregulated inhibitory factors on the surface of various immune-cell populations constitute mechanisms contributing to tumor development.

2.3.1. Endothelin Receptors. Endothelin receptor type A (ET_AR) and type B (ET_BR) are GPCRs that belong to the endothelin system. For a more extensive review, see

Bagnato and Rosanò [88]. Endothelins, the corresponding ligands of the endothelin receptors, are produced in a wide variety of cells, ranging from endothelial to smooth-muscle cells. Synthesis and secretion of endothelin-1 (ET-1), the corresponding ligand of ET_AR, in these cells can be induced by a large array of stimuli within minutes. ET-1 is not stored in the secretory granules of the endothelial cells [89]; therefore, its production translates to high ET-1 plasma levels. Upon binding of its correspondent ligand ET1 located in the plasma, ET_AR promotes vasoconstriction and tumor cell proliferation through a phospholipase C dependent mechanism [90]. On the other hand, ET_BR was shown to regulate T cell adhesion and tumor homing via NO and ICAM-1 [91]. Whether these actions are mediated by ET_BR interaction with ET-1 or one of the other endothelin ligands still remains to be unraveled. In the context of tumor immunology, expression of ET_AR has been reported in prostate cancer patients with bone metastasis [92] and HPV-induced neoplasia [93, 94], whereas ET_BR expression was associated with the absence of tumor infiltrating lymphocytes and decreased survival time of ovarian cancer patients [91]. Also, upregulation of ET_BR in patients with vulvar squamous cell carcinoma has been correlated with tumor progression and early metastasis [95].

2.3.2. Negative Regulatory Factors. Programmed death-1 (PD-1), a member of the CD28 superfamily of T cell regulators [96], is not only a negative regulator of antitumor immunity, but exhibits a broader expression and function, since PD-1 knock-out mice have been shown to develop glomerulonephritis [97] and cardiomyopathy [98]. Expression of PD-1 can be transiently upregulated on the surface of activated $CD4^+$ and $CD8^+$ T cells, B and NKT cells and DCs [99]. Also, high levels of PD-1 have been found on chronically activated $CD8^+$ T cells and during chronic infections [100]. PD-1 has two corresponding ligands, PD-L1 and PD-L2, members of the B7 family [101]. Within the context of tumor immunology the ligand PD-L1, which presents an almost ubiquitous expression profile, is most relevant. Coinhibitory signaling via PD-L1 (but not PD-L2) is necessary for conversion of naïve $CD4^+$ T cells to adaptive $CD4^+FoxP3^+$ Tregs. The PD-1/PD-L1 signaling pathway is viewed as yet another immune escape mechanism of solid tumors [102], due to its capacity to inhibit T cell activation [103] through various downstream signaling effects. Although not as disputed as the PD-1/PD-L1 system, the lymphocyte-activation gene (LAG-3), member of the immunoglobulin superfamily and expressed on the surface of activated regulatory $CD4^+$ and $CD8^+$ T cells, B cells and NKT cells have also been shown to contribute to tumor immunesuppression, as Tregs from LAG-$3^{(-/-)}$ mice present reduced regulatory activity [104].

2.3.3. Secondary Contributive Mechanisms. Other contributive mechanisms of tumor development involve blockade of the granzyme B/perforin pathway by overexpression of the serine protease inhibitor PI-9/SPI-6 [105], modifications in the antigen presentation system [106], developed resistance of tumor cells to apoptosis, and expression of indoleamine 2,3-dioxygenase (IDO) by the tumor or host stromal cells [107].

A large array of various tolerance and antitumor Immunosuppression mechanisms contribute to orchestrating tumor growth and progression. Therefore, effective mono- or polymodality strategies to improve homing and activity of immune effector cells to tumors need to be developed in order for cancer immunotherapy to succeed. A detailed summary of immunotherapeutic strategies developed so far and their corresponding efficiency will be presented in the next section of this review.

3. Shifting the Balance: Strategies to Improve Homing and Activity of Immune Effector Cells to Tumors

To counteract the numerous mechanisms of tumor immune evasion, an ever increasing number of strategies aimed at improving both innate and adaptive antitumor immunity has been developed over time. Based on their overall target aim, these strategies can be categorized as those which attempt to increase homing of effector T cells to tumors and those that, directly or indirectly, increase antitumor activity of intratumoral effector T cells, either by overcoming tumor-induced tolerance or by overriding the immune-suppression mechanisms imposed during tumor development.

3.1. Increased Homing. Due to the large variety of escape mechanisms developed by the tumor microenvironment and the tumor itself, proper trafficking of effector T cells into the tumor may not always occur. An impaired trafficking of these effector cells to the tumor site will give rise to a negative regulatory process, leading to tumor development and progression. Thus, strategies to block this process and enhance homing of effector cells to tumors are of crucial importance for fighting tumor progression. The most widely used strategies to increase recruitment of effector T cells to tumors aim at targeting both the intrinsic alterations of the tumor cells and the extrinsic alterations induced at the level of effector cell populations. These encompass local tumor irradiation, blockade of endothelin receptors, and effector CTL antibody-targeting and taxane-based chemotherapy.

3.1.1. Local Tumor Irradiation. Within the clinical setting, local or total body irradiation is frequently used as adjuvant therapy, in association with other therapies such as surgery, hormonal therapy [108], or bone-marrow transplantation. Evidence is accumulating that local tumor irradiation is able to modulate expression of receptors and cytokines by cancer and stroma cells, resulting in tumor microenvironment changes that can be used for increasing the effects of immune therapy [109, 110]. These changes seem to facilitate recruitment of effector T cells to tumors via two distinct mechanisms: first, by promoting vasculature normalization [111] and second, by stimulating overexpression of endothelial adhesion molecules, such as VCAM-1 [112]. More recent studies indicate that irradiation induces

chemokines involved in recruitment of effector T cells, thus converting tumors into "inflamed tissue", susceptible to the effector phase of the antitumor immune response [113]. For example, a recent study performed by Quezada et al. in which polyclonal $CD4^+$ and $CD8^+$ T cells, harvested from mice previously treated with anti-CTLA-4 and depleted of Tregs, were adoptively transferred into irradiated mice bearing large tumors indicated increased protection against tumor outgrowth [114]. The result seemed to be at least partly due to irradiation-induced overexpression of ICAM and VCAM by the tumor vasculature and increased infiltration of effector T cells to the tumor site. In our hands, local irradiation of TC-1 (HPV transformed) tumor bearing mice with different irradiation dosages induces increased natural infiltration of both $CD8^+$ effector T cells and $CD8^+$ specific CTLs. Also, combination of local TC-1 tumor irradiation with adoptive transfer of *in vitro* restimulated CFSE-labeled specific CTLs lead to a significantly increased homing of the specific CTLs to the tumor site (Draghiciu O, Walczak M, Nijman HW and Daemen T, unpublished data).

3.1.2. Endothelin Receptors Blockade. Various studies demonstrate that endothelial cells from a variety of human cancers overexpress the endothelin receptors. Hence, blockade of these receptors seems to be a promising strategy for delaying tumor development or stopping tumor-cell proliferation. In fact, selective ET_AR blockade by the experimental drug atrasentan has been shown to delay progression of hormone-refractory prostate adenocarcinoma [115] and enhance the effect of paclitaxel/docetaxel used for treatment of prostate cancer [116] in patients. In a mouse model of HPV-induced cervical carcinoma, blockade of ET_AR caused inhibition of tumor growth [94]. Although it can be hypothesized that the effect of ET_AR blockade on tumor growth is mediated by an increase in T cell homing to the tumor site, further studies need to be performed to elucidate the underlying mechanisms. On the other hand, in the context of ovarian and also other cancers, overexpression of ET_BR was associated with the absence of tumor-infiltrating lymphocytes and short patient survival time [117]. Moreover, it was shown that interaction between ET_BR and its corresponding ligand ET-1 induces downregulation of ICAM expression, an effect rescued by administration of the small molecule inhibitor BQ-788. Neutralization of ET_BR by administration of the inhibitory peptide BQ-788 suppressed intercellular communication and cell growth in melanoma nude mice [118] and significantly increased T cell homing to tumors *in vivo* [117].

3.1.3. Antibody-Mediated Targeting of E ector CTLs. Monoclonal antibody therapy is a method most commonly used to functionally inactivate or deplete suppressive immune populations such as MDSCs or Tregs (see Section 3.2.1). However, various studies using bispecific monoclonal antibodies suggest that this approach is a useful tool with a larger therapeutic applicancy. Effector CTL targeting aims at overcoming extrinsic tumor-induced tolerance by making use of bispecific monoclonal antibodies. These particular antibodies are directed against antigens expressed by both activated T cells and tumor cells and display potent *in vitro* [119] and *in vivo* [120] effects against tumor cells. In a severe combined immunodeficiency deficient (SCID) mouse model, this potent antitumor activity has been enhanced even further, due to inhibition of apoptosis of antibody-targeted cytotoxic T cells by costimulation with an anti-CD28 monoclonal antibody [121].

3.1.4. Taxane-Based Chemotherapy. Another relevant tool to increase homing of effector T cells to tumors is chemotherapy with mitotic inhibitors, such as taxanes. Originally, taxanes have been reported to induce mitotic inhibition through disruption of microtubule functionality. Other studies demonstrate their capacity to bind to and block the function of antiapoptotic molecules expressed on the surface of tumor cells, like Bcl-2 [122], thus inducing programmed tumor cell death. A recent study performed by Dirkx and colleagues aimed at investigating whether inhibition of angiogenesis could contribute to overcoming tumor escape from immunity. The results of this study clearly indicated that the angiogenesis inhibitor paclitaxel was capable of increasing leukocyte rolling on the tumor wall vessel and thus infiltration of circulating effector T cells to the tumor [123].

3.2. Increased Activity. Targeting homing of immune effector cells to the tumor site may not solve the problem of eradicating tumor development, as cells that do effectively home to tumor metastases are often found to be dysfunctional. These findings point towards existence of various immunosuppressive mechanisms acquired by the tumor microenvironment in order to fight immune-induced cancer regression. T cell anergy due to extrinsic suppression by regulatory cell populations, inhibition by ligands such as PDL-1, the action of inhibitory factors such as TGF-β, and metabolic dysregulations by enzymes such as indoleamine-2,3-dioxygenase (IDO) have all been implicated in generating this suppressive microenvironment. Effective strategies aimed at increasing activity of T cells that migrate to the tumor site address both inhibition of tolerance and restriction of immunosuppression induced by the tumor microenvironment. For targeting the inhibition of the above described negative regulatory mechanisms, several strategies have been employed over time. Some of the strategies most widely and successfully used in both tumor mouse models and cancer patients will be addressed.

3.2.1. Suppressive Immune Populations: Depletion or Functional Inhibition? One commonly used mechanism of targeting innate as well as adaptive immunity for increasing antitumor activity of effector T cells is depletion of suppressive immune populations. A less intrusive alternative to immune depletion, widely applied as it has been shown to lead to tumor regression [124], consists of manipulating the immune suppressive functions of MDSCs or Tregs. However, functional inhibition of immune suppressive cells, especially directed towards complex and versatile cell populations such as MDSCs, may not be the most suited approach as it is very

likely to lead to development of new inhibitory properties causing *de novo* immunosuppression of the previously restored antitumor immune response.

Different depletion methods, with specificity for the targeted immune population at hand, have been developed over time. Regarding TAMs, selective depletion is promoted by IL-15/TGF-α in human primary colorectal adenocarcinomas [125]. Although TAMs depletion can be achieved by different approaches, such as blockade of TAMs chemoattractant chemokines (e.g., blockade of the chemoattractant CCL-2 with the inhibitor molecule bindarit [126] or vaccination with a legumain-based minigene vaccine [127]), the most efficient depletion method involves the usage of clodronate-liposomes. Clodronate-liposomes are artificial spheres formed by dispersion of phospholipid molecules into an aqueous solution of clodronate bisphosphonate. Intraperitoneal or subcutaneous administration of clodronate liposomes induced efficient depletion (75%–92%) of TAMs in both murine teratocarcinoma and human rhabdomyosarcoma mouse tumor models [128] and in a mouse model of human cervical carcinogenesis, respectively [129]. On the other hand, depletion of MDSCs was achieved either by treatment with tyrosine kinase inhibitors, such as sunitinib [130, 131], which also induced reversal of Treg elevation or by treatment with inhibitors of DNA replication, such as 5-fluorouracil [132] or gemcitabine [133]. Also, nTreg depletion was obtained in animal models by administration of anti-CD25 monoclonal antibodies before inoculation of tumor cells [134]. In line with this approach, it was recently reported that selective depletion of FoxP3$^+$ Tregs by using transgenic DEREG (depletion of regulatory T cells) mice, in combination with therapeutic vaccination against melanoma, greatly enhanced the antitumor effect [135]. However, other studies in this direction indicate that this combinatorial approach consisting of Tregs depletion and vaccination cannot be generalized for obtaining potent antitumor effects. Depletion of Tregs by treatment with the novel antifolate receptor 4 antibody did not enhance the immune response induced by SFVeE6,7 immunization in a mouse model of cervical carcinoma [136].

Functional inhibition of immunesuppressive properties of negative regulatory cell populations is yet another approach towards improving antitumor immunity. One very good example in this direction is constituted by functional Treg inhibition. Blockade of the main inhibitory effector T cell signal CTLA-4, highly expressed on the surface of Tregs, by using anti-CTLA-4 monoclonal antibodies, has been shown to neutralize Tregs mediated suppression of effector T cells [137]. In a similar manner, GITR blockade with mono- or polyclonal antibodies also neutralized nTregs mediated suppression *in vitro* [138]. Although these suppressive strategies have been proven efficient, positive approaches have also been successfully employed: stimulation of human nTregs through TLR8 has been shown to reverse the inhibitory functions of these cells, via signaling through the TLR8-MyD88-IRAK4 pathway, thus increasing antitumor immunity [139]. However, taking into account the phenotypical and immunesuppressive heterogeneity of MDSCs, functional inhibition of these cells seems to be a more challenging matter. Initially, the suppressive activity of MDSCs has been correlated with the metabolism of L-arginine, the substrate of both iNOS and arginase-1. Accordingly, administration of cyclooxygenase 2 inhibitors has been shown to block production of prostaglandin E2 and thus induce a signaling cascade leading to downregulation of both arginase-1 and iNOS expression on MDSCs. In its turn, this downregulation was associated with increased efficacy of antitumor immunotherapy [140, 141]. However, nowadays an increasing body of evidence points towards ROS and peroxynitrite production as one of the main mechanisms of MDSC-induced effector T cell inhibition. ROS induces effector T cell anergy by direct damage at the DNA level, whereas peroxynitrite is hypothesised to directly nitrosylate intracellular T cell tyrosine to nitrotyrosine, thereby inducing CD8$^+$ T cell unresponsiveness [142]. Inhibition of ROS production has been shown to abrogate the MDSCs suppressive effects *in vitro* [45]. Also, *in vitro* treatment of isolated MDSCs with the anti-inflammatory triterpenoid drug CDDO-Me reduced both ROS and peroxynitrite levels, whereas *in vivo* administration of CDDO-Me to tumor bearing mice lead to a significant decrease in tumor size [124]. The reduced tumor size after CDDO-Me treatment could partly be explained by decreased ROS and peroxynitrite production.

Other effective methods of MDSCs manipulation towards a better outcome of the antitumor immune response include induction of MDSCs differentiation into myeloid DCs that have lost their suppressive activity, by administration of all-trans retinoic acid [143] and inhibition of MDSCs maturation from precursors, by usage of selective inhibitors of the STAT3 maturation pathway [144].

3.2.2. Blockade of Negative Regulatory Factors. Within the context of tumor development and progression, overexpression of negative regulatory factors, such as PD-1 and LAG-3, has often been correlated with chronically activated and nonfunctional CD8$^+$ T cells. Hence, blockade of either of these two factors could be an efficient strategy to induce tumor regression. In this context, PD-1 blockade has been shown to increase the induction of effector T cells in the spleen, prolong T-cell proliferation, and enhance recruitment of effector T cells to tumor sites [145]. In multimodality therapy regimens, PD-1 blockade increased therapeutic efficacy of total body irradiation and DCs transfer therapy [146]. Also, antibody blockade of LAG-3 in two murine models of self- and tumor-tolerance increased the accumulation and effector function of antigen-specific CD8$^+$ T cells [147]. Thus, combination of mAb therapy against PD-1 or LAG-3 with vaccination strategies has been recently demonstrated to restore the functions of tolerized antigen-specific CD8$^+$ T cells [148].

3.2.3. Blockade of TGF-β Induced Signaling. Several approaches have been employed to induce high avidity effector T cells in an attempt to target the inhibition of tumor-induced tolerance. One such approach involves blockade of TGF-β induced signaling. In a xenograft mouse

model of prostate cancer, transfer of tumor-reactive TGF-β-insensitive $CD8^+$ T cells leads to a 50% decrease in average tumor weight, when compared with tumors of mice which underwent transfer of naïve $CD8^+$ T cells [149]. Another approach aimed at manipulating TGF-β to improve antitumor immune responses involves generation of TGF-β insensitive DC vaccines. Transduced DCs, which have been rendered insensitive to TGF-β, maintain their normal phenotype, present upregulated expression of surface costimulatory molecules (CD80/CD86) and induce potent tumor-specific cytotoxic T lymphocyte responses *in vivo* [150].

3.2.4. Blockade of Indoleamine 2,3-Dioxygenase (IDO). IDO is an enzyme constitutively expressed by both various tumor cell lines and diverse human tumors, such as cervical, pancreatic, and colorectal carcinomas. IDO-expressing tumors were shown to block antigen-specific T cell proliferation, thus mediating the process of tumor immune escape [151]. Plasmacytoid dendritic cells (pDCs) resident in tumor-draining lymph nodes were also shown to express high levels of functionally active IDO, which mediated suppression of reactive T lymphocytes. Since IDO catalyzes the first step in the metabolism of tryptophan, IDO activity in tumors and pDCs altogether can be inhibited by various tryptophan analogues such as 1 methyl-tryptophan. Inhibition of IDO activity in pDCs by administration of 1 methyl-tryptophan leads to reversal of T cell suppression [152].

3.2.5. CTLs Manipulation Strategies. So far, proper targeting and usage of different methods to increase host antitumor immune responses have been discussed. However, one other option to consider when aiming for tumor eradication consists of developing strategies which target the expansion and activation of the effector cell populations themselves. Within this context, adoptive cell therapy or other direct CTL manipulation strategies (such as cisplatin treatment) might just do the job.

(a) Adoptive Cell Therapy (ACT). Adoptive T cell therapy is a very widely used clinical method employed for cancer treatment. More extensive reviews regarding ACT have been written over time [153–155]; here, some aspects of ACT relevant to the induction of tumor regression will be briefly highlighted. T-cell-based ACT constitutes another approach of increasing effector T-cell number and activity, by *in vitro* expansion of a patient's own CTLs and *in vivo* reinfusion of the expanded CTLs into the patient himself, associated or not with concomitant exogenous administration of IL-2. ACT can be viewed as a method of indirectly manipulating the immune system towards the induction of a new CTL population. In many cases, lymphodepletion is required before CTL reinfusion, in order to eliminate Tregs or other competing own lymphocytes. Several studies have already been performed in this direction and the highest efficiency of ACT has been reached in patients with metastatic melanoma [156]. In the clinical setting, ACT has been used either as mono- (e.g., expansion and conversion of Tregs [157]) or in polymodality treatments, in combination with gene therapy [158] or total body irradiation to achieve lymphodepletion [159–161].

(b) Platinum-Based Chemotherapy. Originally, it was shown that platinum-based chemotherapy leads to tumor cell apoptosis by binding to and causing DNA crosslinking. Recent studies aiming to completely unravel the effects of platinum-based chemotherapy on $CD8^+$ T cell mediated immunity reported that cisplatin greatly enhances E7-specific $CD8^+$ T cell immunity induced by DNA vaccination in TC-1 tumor bearing mice [162]. Also, combined chemotherapy treatments with the two platinum-based drugs paclitaxel and carboplatin resulted in improved survival in advanced ovarian cancer patients [163]. The observed improved survival might be explained by a synergistic effect of combined therapy, leading to induction of higher cytotoxic T lymphocytes frequencies. However, platinum-based drugs do not constitute the only chemotherapeutics capable of enhancing the antitumor immune function of effector T cells. The alkylating drug cyclophosphamide (CTX) is yet another agent widely used in the chemoimmunotherapy of tumors. CTX has been shown to synergize with exosome-based vaccines by abolishing the Tregs suppressive function and enhancing the vaccine-induced CTL responses in murine tumor models [164]. Also, CTX treatment has been shown to induce differentiation of $CD4^+$ Th17 cells in cancer patients [165].

3.2.6. Therapeutic Vaccination. Although the mechanisms by which chronic viruses or bacteria infections promote cancer are quite diverse, a common feature is given by the fact that development of cancer takes place in the setting of chronic infections [166]. To this end, prophylactic immunization strategies have been developed to reduce cancer burden and a very suitable example is given by production of the two prophylactic vaccines Gardasil and Cervarix for HPV-induced cervical cancer. Regarding therapeutic vaccination, Provenge, containing the active substance sipuleucel-T, is the first therapeutic cancer vaccine that demonstrated effectivenessby prolonging life of patients with metastatic prostate cancer [167]. Sipuleucel-T is constituted by peripheral blood mononuclear cells activated *ex vivo* by the recombinant human PAP-GM-CSF fusion protein (prostatic acid phosphatase-granulocyte-macrophage colony-stimulating factor [168]).

Another therapeutic vaccine, which induced both strong, long-lasting CTL responses in a mouse model of cervical carcinoma and effective eradication of established tumors of HPV-transformed cells [169, 170], is the recombinant Semliki Forest virus vaccine (rSFV). Constituted of a fusion protein of HPV16 E6 and E7 (SFVeE6,7), this vaccine was able to induce specific CTL activity in immune-tolerant, E6/E7 transgenic mice [171]. A comparative study between the prime-boosting efficacy of SFVeE6,7 and that of the recombinant adenovirus type 5 vector expressing the same antigen construct (Ad-eE6,7) revealed that SFVeE6,7 vaccination lead to higher precursor CTL frequencies and

activity when compared to Ad-eE6,7 vaccination. The efficacy of SFVeE6,7 vaccination in murine tumor treatment experiments was significantly higher than that of the Ad-eE6,7 counterpart [172]. Also, low doses of IL-12 expressed by a SFV virus vector (SFV-IL12) augmented the antigen-specific and antitumor responses induced by the virus vector alone [173]. More recent studies showed that the rSFV vaccine induces strong CTL responses in both homologous [174] and heterologous [175] prime-boost immunization regimens in a mouse model of cervical cancer. However, contrary to the excellent therapeutic antitumor responses observed in animal tumors, the clinical results in patients are modest. Explanations for this outcome may be either insufficient activation of antigen-specific immune effector cells or development of immune-suppression mechanisms. For this purpose, development of new multimodality strategies in which vaccination therapies are combined with effective antitumor approaches aimed at increasing homing and activity of immune effector cells to tumors is of crucial importance and thus, an important step forward in cancer immunotherapy.

4. Concluding Remarks

In the last few decades, major progress has been achieved within the field of cancer immunotherapy. However, despite this progress, the outcomes of clinical trials performed so far are significantly lower than expected. Contrary to the excellent therapeutic antitumor responses observed in animal tumors, the clinical results in patients are modest. Explanations for this outcome may be either insufficient homing and activation of antigen-specific immune effector cells within the tumor or development of immune-suppressive mechanisms, capable of inhibiting their cytolytic activity. Both recent experimental studies and emerging clinical trials indicate towards development of good vaccination strategies, leading to generation of high levels of effector T cells with a proper phenotype and specificity, as a possible answer to the problem. A desirable, highly effective vaccination strategy should accomplish two purposes. On one hand, it should aim at increasing both the recruitment of antigen-specific effector T cells to the tumor site and their intratumoral arrest for the time necessary to exert their antitumor activity. For this purpose, combination of vaccination regimens, leading to induction of high levels of antigen-specific effector T cells, with ways to enhance homing of these cells to the tumor site, such as local tumor irradiation, endothelin B receptor blockade, antibody-mediated targeting of effector CTLs, or taxane-based chemotherapy, could be a promising strategy. On the other hand, targeting only the homing of vaccine-induced effector T cells to the tumor site might not be enough. We may speculate that once these cells have reached the tumor, they can be anergized or tolerized by diverse immune-suppressive mechanisms developed by the tumor itself or by secondary immune-suppressive populations. To counteract this effect, strategies which aim at maintaining or potentiating the activity of these intratumoral antigen-specific effector T cells, such as depletion or functional inhibition of immune-suppressive populations, blockade of negative regulatory factors, CTLs manipulation methods, or therapeutic vaccination are stringently necessary.

Concluding, development of new multimodality strategies in which vaccination therapies are combined with effective antitumor approaches aimed at increasing homing of immune effector cells to tumors and their intratumoral activity is of crucial importance and might represent the next step forward in cancer immunotherapy.

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Review Article

Interferon Lambda: A New Sword in Cancer Immunotherapy

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The discovery of the interferon-lambda (IFN-λ) family has considerably contributed to our understanding of the role of interferon not only in viral infections but also in cancer. IFN-λ proteins belong to the new type III IFN group. Type III IFN is structurally similar to type II IFN (IFN-γ) but functionally identical to type I IFN (IFN-α/β). However, in contrast to type I or type II IFNs, the response to type III IFN is highly cell-type specific. Only epithelial-like cells and to a lesser extent some immune cells respond to IFN-λ. This particular pattern of response is controlled by the differential expression of the IFN-λ receptor, which, in contrast to IFN-α, should result in limited side effects in patients. Recently, we and other groups have shown in several animal models a potent antitumor role of IFN-λ that will open a new challenging era for the current IFN therapy.

1. Introduction

Despite the early discovery of interferon (IFN) in 1957, IFN lambdas were just identified during the recent years and classified as a new group, type III IFN. In human, 3 distinct proteins called IFN-λ1, IFN-λ2, and IFN-λ3 have been identified [1, 2]. They are also named interleukin-29 (IL-29), IL-28A, and IL-28B, respectively [3]. The members of this new IFN family were found to interact through unique receptors that are distinct from type I (IFN-α/β) and type II (IFN-γ) IFN receptors. The receptor for type III IFN is composed of the unique IFN-λR1 chain also called IL-28AR and the IL-10R2 chain, which is shared with IL-10, IL-22, and IL-26 receptor complexes. Although type III IFNs bind to a specific receptor, the downstream signaling is similar to that induced by type I IFNs. Both type I and type III IFNs stimulate common signaling pathways, consisting of the activation of Jak1 and Tyk2 kinases and leading to the activation of IFN-stimulated gene factor 3 (ISGF3) transcription complex. ISGF3 is composed of STAT1 and STAT2 and the interferon regulatory factor IRF9 (ISGF3-γ or p48) (Figure 1). Although there are three genes encoding highly homologous but distinct human IFN-λ proteins (IFN-λ1, IFN-λ2, and IFN-λ3), our search of the mouse genome revealed the existence of only two genes, representing mouse *IFN-λ2* and *IFN-λ3* gene orthologues, located in chromosome 7 and encoding intact proteins. The mouse *IFN-λ1* gene orthologue is a pseudogene containing some variations in addition to a stop codon in the first exon and does not code for an active protein [4]. We have cloned the mouse IFN-λs (mIFN-λ2 and mIFN-λ3) and IFN-λ receptor (mIFN-λR1) orthologues and found them to be quite similar to their human counterparts. Experiments showed that similar to their human counterparts, mIFN-λ2 and mIFN-λ3 signal through the IFN-λ receptor complex, activate ISGF3, and are capable of inducing antiviral protection and MHC class I antigen expression in several cell types. The results showed that murine type III IFNs (IFN-λs) engage a unique receptor complex, composed of IFN-λR1 and IL-10R2 subunits, to induce signaling and biological activities similar to those of type I IFNs. Interestingly, in contrast to type I and type II IFNs, type III IFNs demonstrate less species specificity.

2. Biological Properties of IFN-λ

2.1. Restrictive Cell Response to Type III IFN (IFN-λs). Although type I and type III induced similar cell signaling,

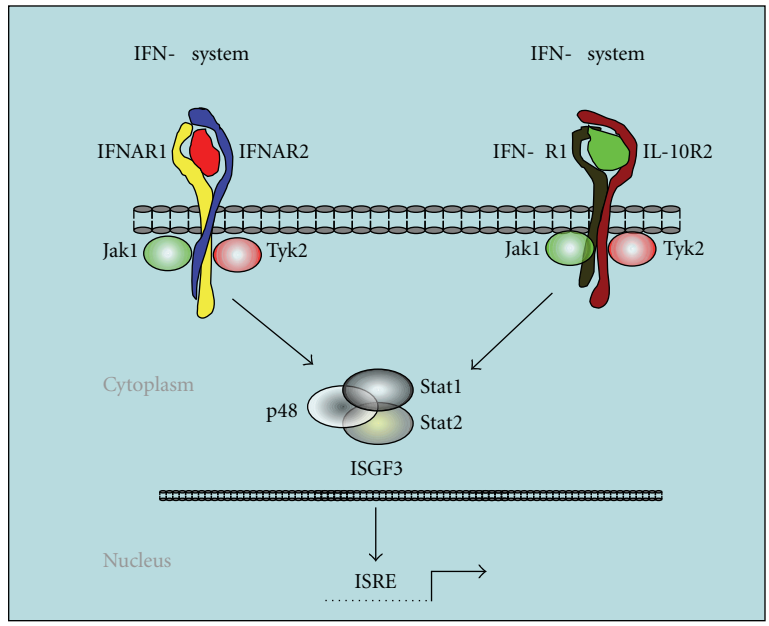

Figure 1: IFN-α and IFN-λ receptor systems and cell signaling. IFN-α and IFN-λ interact with distinct receptors, but the downstream signaling is similar. IFN-α interacts with receptors composed of IFNAR1 and IFNAR2, and IFN-λ interacts with a receptor composed of a specific chain, IFN-λR1, and IL-10R2, a shared subunit with IL-10, IL-22, and IL-26. Both IFNs lead to the activation of the Jak kinases (Jak1 and Tyk2) and the formation of the transcription-complex-designated IFN-stimulated gene factor 3 (ISGF3), which includes p48, Stat1, and Stat2. ISGF3 complex binds to the IFN-stimulated response element (ISRE) and induces gene transcription.

the intensity of cell signaling as measured by STAT1 activation appeared to be significantly lower for type III IFNs [4]. In comparison with type I IFN, only restricted cell types respond to type III IFN (Figure 2). Interestingly, we did not find a strict correlation between the intensity of cell signaling induced by IFN-λ and the level of biological activity. For example, in B16 melanoma cells, although IFN-λ induced a very weak STAT1 activation in comparison with IFN-α, we observed a robust stimulation of MHC class I expression at the cell surface, indicating the potential contribution of cell-specific modulators of the IFN-λ activity.

Antiviral studies performed *in vitro* and *in vivo* have shown that both IFN-α and IFN-λ contribute to the overall host antiviral defense system [2, 3, 5–8]. It has been demonstrated that IFN-λ induces antiviral activity against VSV (vesicular stomatitis virus) and EMCV (encephalomyocarditis) in many human cell lines [2, 3, 9, 10]. However, by using different mouse models of viral infection, Ank et al. demonstrated that IFN-λ was effective against DNA virus, simplex virus 2 HSV2 but not RNA viruses such as EMCV and lymphocytic choriomeningitis virus LCMV [6]. Several other studies demonstrated that type III IFNs can also inhibit replication of hepatitis C virus (HCV) and hepatitis B virus (HBV) *in vitro* [10–14]. These studies were important since they underlined the fact that IFN-λ could be used as an alternative to IFN-α for HCV patients who are resistant to IFN-α treatment. It has been reported that IFN-λ has the ability to inhibit human immunodeficiency virus type 1 (HIV-1) infection of blood monocyte-derived macrophages that expressed IFN-λ receptors [15] and the herpes simplex virus type 1 (HSV1) infection of human astrocytes and neurons [16]. However, in most other cases, the antiviral potency of IFN-λ against several viruses seems to be lower than that of IFN-α [2, 3, 8, 9, 13, 17]. In addition, IFN-λ and IFN-α may induce distinct signal transduction and gene regulation kinetics [13, 18].

Moreover, type I IFN-α activates a plethora of innate and adaptive immune mechanisms that help eliminate tumors and viral infections. IFN-α immunoregulatory functions include major histocompatibility complex (MHC) class I expression in normal and tumor cells, activation of NK cells, dendritic cells (DCs), and macrophages, resulting in the promotion of adaptive immune responses against tumors and virally infected cells [19, 20]. The role of IFN-λ in the immune system is currently being investigated by several groups. So far, data suggests that IFN-λ exerts immunomodulatory effects that overlap those of type I IFN. It has been recently demonstrated that human IFN-λ1 (IL-29) modulates the human plasmacytoid DCs function and cytokine response [21, 22]. IFN-λ1 treatment of whole peripheral blood mononuclear cells (PBMCs) upregulated the expression of IL-6, IL-8, and IL-10 but not IL-1 or TNF. This IFN-λ-induced cytokine production was inhibited by IL-10. By examination of purified cell populations, it was also shown that IFN-λ1 activated monocytes, rather than lymphocytes, resulting in the secretion of the above panel of cytokines, suggesting that IFN-λ1 may be an important activator of innate immune responses particularly at the site of viral infections [21]. IFN-λ1 was also shown to possess immunoregulatory functions on T helper 2 (Th2) responses by markedly inhibiting IL-13. However, only moderate effect was observed on IL-4 and IL-15, the

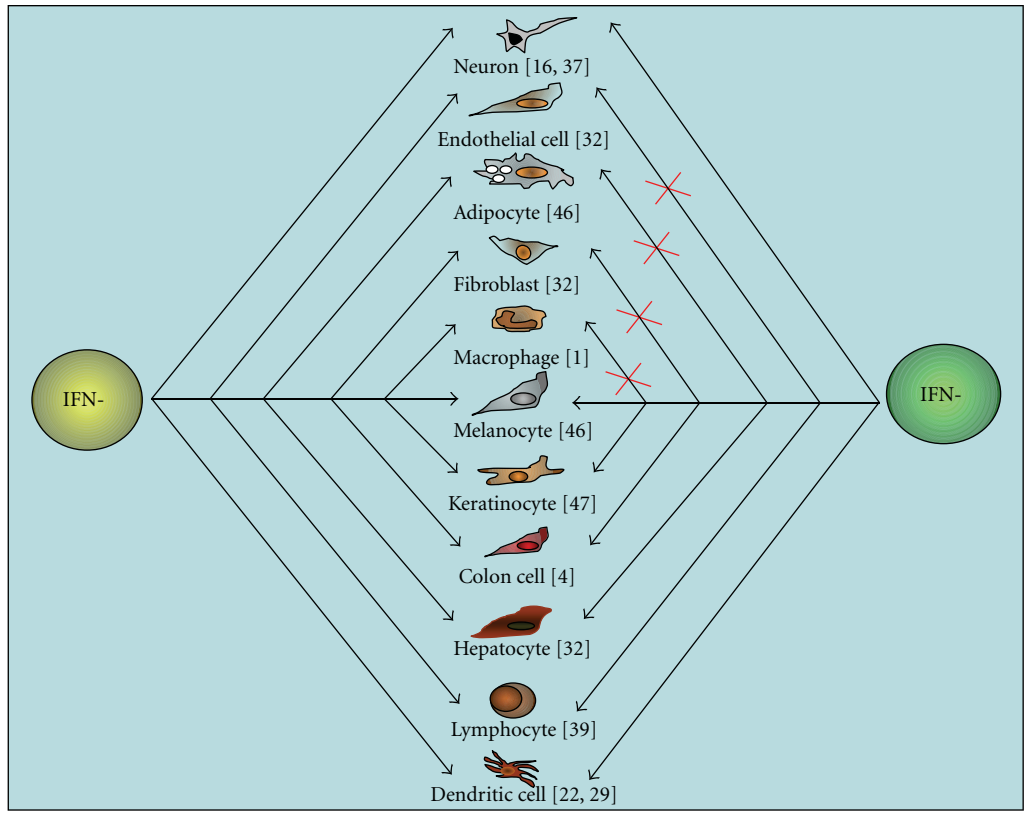

FIGURE 2: Cellular targets for type I and type III IFNs. Response to IFN-α and IFN-λ in cells from different origins in human. The IFN response was assessed by measuring the IFN-induced cell signaling (Stat activation) and cell activity (MHC class I antigen stimulation). In contrast to IFN-α, only restricted cells respond to IFN-λ, including epithelial-like cells, forming the major organs of the body.

other important cytokines in the Th2 response [23–25]. This immunoregulatory function was enhanced through the expression of IFN-λR1 on $CD4^+$ T cells [23]. These findings correlate with data suggesting that IFN-λ may have an immunoprotective role against asthma, the allergy disease caused by an exaggerated Th2 response [9, 26, 27].

Similar to IFN-α, IFN-λ produced by DCs, in response to toll-like receptor (TLR) stimulation, was found to have specific effects on DC differentiation and maturation [28], which include only partial maturation of DCs, upregulation of MHC class I and II molecules, and no induction of co-stimulatory molecules [9, 29]. During their differentiation from monocytes, DCs acquire IFN-λ responsiveness through the expression of IFN-λR1. Interestingly, DCs treated with IFN-λ promoted the generation of tolerogenic DCs and the IL-2-dependent proliferation of Foxp3-expressing $CD4^+CD25^+$ regulatory T cells (Tregs) [29]. More recently, Morrow et al. have demonstrated, through DNA vaccination with plasmids encoding IFN-λ3 (IL-28B) and IL-12, that IFN-λ3, just like IL-12, is able to enhance adaptive immunity. However, in contrast to IL-12, IFN-λ3 reduces regulatory T-cell populations. They also showed that unlike IL-12, IFN-λ3 is able to increase the percentage of splenic $CD8^+$ T cells in vaccinated animals and that IFN-λ3 can completely protect mice from death following a lethal influenza challenge [30]. These studies altogether highlight the strong candidacy of IFN-λ as a potential novel immunotherapeutic agent.

In addition to antiviral and immunomodulatory activities, type I IFNs demonstrate antiproliferative activities in most cell lines, while this activity seems to be restricted with IFN-λs [9, 17]. Type I IFNs have been shown to induce apoptosis in tumor cells, yet the molecular mechanisms mediating cell death in response to these IFNs remain to be fully explained. By binding to their corresponding cellular receptor complexes, IFNs induce a quick and potent signaling which leads to the expression of more than 300 IFN-stimulated genes (ISGs) [13, 31, 32]. Many ISGs encode proteins that have been implicated in apoptosis [33, 34]. Unlike IFN-α, IFN-λs do not inhibit the proliferation of several cell lines including the Daudi cells (a B-lymphoblastoid cell line from Burkitt's lymphoma), which strongly respond to type I IFNs in an antiproliferative assay [2, 3, 10, 17]. However, it was demonstrated that IFN-λs do inhibit the proliferation of few tumor cell lines, such as the LN319 human gliobastoma cell line [17] and of cells constitutively expressing high levels of IFN-λR1 [35]. The antiproliferative effects of IFN-λ have been demonstrated in various tumor cell lines that express ectopic or endogenous IFN-λ receptors [17, 36, 37]. Therefore, the ability of IFN-λs to induce antiproliferative activity in cells depends on the level of IFN-λR1 expression.

It has been recently reported that IFN-λ signaling in colorectal adenocarcinoma HT29 cells led to caspase activation, externalization of phosphatidylserine (PS), and DNA fragmentation, resulting in subsequent apoptosis [38]. This study provided evidence for the first time that type III IFNs, alone or in combination with other stimuli, have the potential to induce apoptosis. Moreover, another recent study revealed that IFN-α and IFN-λ differ in their antiproliferative effects and this was correlated with a difference in the duration of JAK/STAT signaling activity between the two IFNs and prolonged ISG expression upon IFN-λ treatment [18]. Using the human keratinocyte HaCaT cell line that expresses receptors for both IFN-α and IFN-λ, they found that IFN-λ induced a more pronounced growth inhibitory effect than IFN-α. IFN-λ was also more efficient than IFN-α in inducing an antiproliferative effect that overlapped with the activation of apoptosis. Prolonged duration of IFN-λ-induced STAT activation, and ISG expression could account for the enhanced antiproliferative and proapoptotic effects observed in HaCaT cells, effects not seen upon treatment with high doses of IFN-α [18]. Interestingly, a study has shown that IFN-λ can induce the growth of human multiple-myeloma cells and antagonize the dexamethasone-induced cell death in these cells [39]. IFN-λ-mediated cell growth of multiple myeloma cells was MAPK dependent [39]. High level of IFN-λ was found in the malignant bone marrow microenvironment, implying that IFN-λ may play a direct role in multiple myeloma development.

2.2. Tissue and Species Specificity of Type III IFN (IFN-λ). By using a plasmid electrotransfer approach, Sommereyns and coworkers reported a differential response to IFN-λ in mice, with a very low response to IFN-λ for the liver, central nervous system, and spleen. However, a high response to IFN-λ was observed in the stomach, intestine, heart, kidney, and lung [40]. The IFN-λ response was restricted to epithelial cells and correlated with the expression of IFN-λR (IL-28Ralpha). Paradoxically in mice, in spite of the epithelial nature of the hepatocytes, the liver expressed low levels of IL-28Ralpha and responded poorly to IFN-λ [8, 40]. However, a significant response to IFN-λ was reported in human hepatocytes [13, 32], suggesting the existence of some variations in the response to IFN-λ between mice and humans, at least in the liver. Although the main IFN-λ targets are the epithelial cells, the presence of potential tissue-specific factors may modulate the IFN-λ response through the IFN-λ receptors. Recently, it has been shown in mice that in contrast to the hepatocytes, prominent response to IFN-λ was observed in intestinal epithelial cells. In comparison with IFN-α, this response is higher and plays a critical role in protecting the intestinal epithelium from viral infection [41], strongly suggesting the prominent role of IFN-λ in organs with mucosal surface at least in mice [6, 42, 43]. In addition to the direct effect of IFN-λ on the mucosal epithelium, local immunomodulations can also be promoted [44].

2.3. Distribution of IFN-λR1 and Responsiveness to IFN-λ. The functional IFN-λR is formed by two chain proteins, IFN-λR1 (also called IL-28Ralpha) and IL-10R2. IFN-λR1 is unique for the IFN-λs, and its tissue distribution is highly restricted. In contrast to IFN-λR1, IL-10R2 is shared by IL-10, IL-22 and IL-26 and ubiquitously expressed in all tissues. Unlike IFN-α, only few cell types respond to IFN-λ (Figure 2). In contrast to the epithelial-like cells, fibroblasts and endothelial cells were completely unresponsive to IFN-λ [4]. Although the hematopoeitic system is not the primary target of IFN-λ, the response of some subpopulations to IFN-λ is not excluded. In mice, we found that IFN-λ induces STAT1 activation in both plasmacytoid and myeloid dendritic cells [45]. These results are in accordance with those obtained by Mennechet and Uzé [29], who proposed the acquisition of an IFN-λ response by monocytes after their differentiation into dendritic cells. Therefore, the response to IFN-λ may be controlled by the induction of the IFN-λR1 expression. Different levels of IFN-λR1 were found in different tissues [40, 43, 46]. The highest levels were found in the gastrointestinal tract and lung. The brain showed the lowest level of receptor expression. The IFN-λR1 expression was also analyzed in different cell types. The expression of cell populations isolated from human skin showed a high expression of IFN-λR1 in keratinocytes and melanocytes. However, dermal fibroblasts, endothelial cells, and subdermal adipocytes did not express significant amounts of IFN-λR1. Significant expression of IFN-λR1 was detected in primary human hepatocytes in comparison with the chondrocytes, isolated from the hyaline cartilage of the knee joint [46, 47]. Although the expression of IFN-λR1 was significantly high in lymphoid tissues, the IFN-λ response was very weak, implying the presence of specific mechanisms in the lymphoid tissues that may inhibit the IFN-λ response. For example, IFN-λR1 levels in B cells are threefold those detected in keratinocytes, which exhibit one of the highest responses to IFN-λ. Witte et al. proposed the potential role of soluble IFN-λR1, highly released by the immune cells, in this weak response to IFN-λ [46].

Although all the IFN-λs interact with the same receptor, IFN-λR1, the binding characteristics for each ligand are still under investigation. In the future, it will be important to analyze the IFN-λ activity in light of the IFN-λ binding to the cells and understand particularly the role of IFN-λ3, which possesses the highest activity as compared with the other IFN-λs [48, 49]. Analysis of the ligand binding in combination with the activity induced by IFN-λ will be also important in understanding the impact of IFN-λ in epithelial cells, particularly in comparison with the immune cells expressing IFN-λR1. Besides several carcinomas, originating from epithelial cells, which respond to IFN-λ, other tumors not arising from epithelial cells may become more sensitive to IFN-λ. It was reported that multiple myeloma cells, which originate from B-cell plasmocytes, showed high binding and response to IFN-λ [39]. Studying the IFN-λ binding in transformed cells versus normal cells may be very helpful for tumor targeting and for the establishment of the optimum dose of IFN-λ to be used for the *in vivo* treatment. IFN-λ can also be used as a drug carrier, to specifically target a drug to tumors expressing high IFN-λ binding sites.

2.4. Antiviral Protection in IFN-Type-III-Deficient Mice. The availability of IFN-λR1 knock-out mice allowed for the investigation of the role of type III IFNs *in vivo*. By using those mice, Mordstein et al. showed for the first time the contribution of IFN-λ in the innate immunity against the influenza virus [8]. Later, they found that IFN-λ played an important role in the defense against other pathogens that infect the respiratory tract, such as the respiratory syncitial virus, the metapneumovirus, and the severe acute respiratory syndrome (SARS) coronavirus. However, the lassa fever virus which replicates in the liver, was not affected by the lack of IFN-λR1 [50]. Although this study clearly demonstrated that IFN-λ played an important role in protecting the respiratory and gastrointestinal tracts against virus infection, in comparison with type I IFN, the protection provided by type III IFN remains limited. However, in combination, type I and type III may provide a better viral protection. When the response to both type I and type III is deficient, the mice are not able to clear the SARS coronavirus from the intestine as compared with mice in which type I or type III remains functional, implying that IFN-λ may strengthen the antiviral activity by acting as a first line of defense for the mucosa [8, 50].

2.5. Clinical Use of Type III IFN. The first use of IFN-λ in the clinic has started for hepatitis C. The phase 1b study has been conducted in patients with chronic genotype 1 hepatitis C virus infection ((HCV) [51]). Pegylated IFN-λ1 in combination or not with ribavirin (RBV, which belongs to a class of antiviral medications called the nucleoside analogues) has been used in this study to assess the efficacy and the potential cytotoxicity. The study was performed in 3 parts. The first part evaluated the pegylated IFN-λ as single agent for relapsed patients after IFN-α-based treatment. The second part concerned the combination of pegylated IFN-λ and RBV in treatment-relapse patients. The third part evaluated pegylated IFN-λ in combination with RBV in treatment-naïve patients. In addition, different doses (from 0.5 to 3 microg/kg) of pegylated IFN-λ were used. Fifty-six patients were enrolled. 24, 25, and 7 patients were used, respectively, for part 1 to 3. The data showed an antiviral activity in all doses of pegylated IFN-λ tested. 29% of treatment-naïve patients achieved rapid antiviral response. As expected, due to the limited IFN-λR1 distribution, the treatment was well tolerated with few adverse effects. Minimal flu-like symptoms and limited hematologic suppression were reported. In summary, the authors concluded that weekly pegylated-IFN-λ with or without daily RBV for 4 weeks is associated with a clear antiviral activity in patients with chronic HCV. However, this study lacks a direct comparison between IFN-λ and IFN-α and the influence of viral and patient genotypes. Now it is well accepted that the response to IFN-α or the natural clearance of HCV infection is depending on single-nucleotide polymorphisms (SNPs), upstream of IFN-λ3, which could be used as biomarkers to help determine the treatment outcome [52]. The first genome-wide association studies (GWAS) in HCV infection were reported by Ge et al. They evaluated the treatment outcome in a group of 1671 patients of mixed ethnicity, receiving pegylated IFN-α and ribavirin. An association was discovered between sustained viral response (SVR) to treatment and a cluster of seven SNPs linked to the IFN-λ3 gene, with the most significant SNP (rs12979860) demonstrating high statistical significance [53]. Many other studies have replicated these findings, demonstrating the high link between IFN-λ3 and treatment outcome [54–61]. However the mechanisms explaining this link remain to be determined. It is not clear yet if this SNP is associated with a constitutive production of IFN-λ that may play a role in HCV clearance and the success of IFN-α treatment. These results also suggest the therapeutic potential of the IFN-α and IFN-λ combination therapy as demonstrated for the hepatocellular carcinoma (HCC) mouse model [62].

3. Emergence of IFN-λ as a New Antitumor Agent

3.1. Characterization of the IFN-λ System and Demonstration of Its Antitumor Activity in a Melanoma Model. Although they engage distinct receptors, IFN-α and IFN-λ induce similar cell signaling (Figure 1). Since IFN-α is widely used in the clinic to treat cancer (Table 1), we have investigated the potential antitumor activity of IFN-λ by using the mouse B16 melanoma model. We have chosen this cancer model because melanoma is a very aggressive cancer, and one of the therapeutic agents frequently used in the treatment of melanoma is IFN-α. Significant improvements in relapse-free and overall survival, with postoperative adjuvant IFN-α therapy, have been reported by large and randomized studies [63–65]. However, the beneficial effect of IFN-α was only obtained when the patients received high doses (20 MIU/m2 intravenously five times per week). Studies with low doses of IFN-α have not shown significant increase in overall survival [66, 67]. Usually, the dose for optimal antitumor activity is higher than the maximally tolerated dose. This dose dilemma profoundly affects the acceptance of IFN-α treatment by both the clinicians and the patients. The adverse effects associated with high doses of IFN-α include myelosuppression and nervous system disorders. These effects often compromise the beneficial antitumor effect, with premature discontinuation of the treatment or the reduction of the dose of IFN-α. Since virtually all the cells of the body respond to IFN-α, it is not surprising that the patients develop numerous side effects. Making a dissection between the beneficial and harmful effects of IFN-α is a very challenging task, which requires more investigation of the interferon system. To investigate the antitumor effect of IFN-λ in melanoma, we have used a gene therapy approach, consisting on the delivery of the IFN-λ gene to tumor cells. Gene transfer into tumor cells is very useful approach to test the effectiveness of cytokines in animal cancer models. This approach does not require production and purification of the protein. The secretion of constant amounts of various cytokines by transduced tumor cells at the site of tumor growth could elicit more

Table 1: Clinical indications of IFNs. IFN-α with different trade names is the most indicated in the clinic. IFN-β is mostly indicated for the treatment of relapsing remitting multiple sclerosis. IFN-γ is only indicated for the chronic granulomatous disease. IFN-λ, the new type of IFN, was tested for patients with chronic hepatitis C.

IFN type	Indications in the clinic
IFN-α	Hairy cell leukemia
	Multiple myeloma
	Chronic myeloid leukemia
	Follicular lymphoma
	Cutaneous T lymphoma
	Kaposi sarcoma
	Melanoma
	Renal cell carcinoma
	Hepatocellular carcinoma
	Condyloma accuminata
	Hepatitis B
	Hepatitis C
IFN-β	Multiple sclerosis
IFN-γ	Chronic granulomatous disease
IFN-λ	Hepatitis C

effective antitumor responses by acting directly on the tumor microenvironment. Another advantage of the cytokine gene transfer into tumor cells versus systemic administration is the potential of inducing the antitumor effect without eliciting the side effects associated with the systemic administration of high doses of cytokines.

To investigate the potential antitumoral role of IFN-λ, we first evaluated the response of B16 melanoma cells to IFN-λ, by analyzing STAT1 activation and MHC class I antigen expression. In comparison with IFN-α, we have found that IFN-λ induces weak STAT1 phosphorylation but strong stimulation of MHC class I antigen expression, indicating a difference between IFN-α and IFN-λ in the link intensity of cell signaling/biological activity. This result warrants further investigation in comparing the response to IFN-α and IFN-λ. By using gene transfer, we engineered B16 cells, which constitutively produced mIFN-λ (B16.IFN-λ cells). In response to their secretion of IFN-λ, B16.IFN-λ cells exhibited constitutively high levels of MHC class I antigen expression. All the C57BL/6 syngeneic mice injected with parental B16 cells developed tumors. However, the constitutive production of mIFN-λ by B16.IFN-λ cells markedly affected tumorigenicity of the cells. B16.IFN-λ cells were either rejected by the host or grew at a slower rate than control parental B16 cells. The antitumor effect of IFN-λ was dose dependent. B16.IFN-λ cells also inhibited the growth of parental B16 cells when both cell types were injected together [4]. We also used the engineered B16.IFN-λ Res. cells, which, in addition to their constitutive IFN-λ secretion, are completely resistant to IFN-λ, as demonstrated by the lack of IFN-λ-induced MHC class I antigen expression. Interestingly, similar to B16.IFN-λ cells, we have found a reduction of the tumorigenicity of B16.IFN-λ Res. cells, implying the involvement of host antitumor mechanisms induced by IFN-λ [4].

Following our report on the characterization of the mouse IFN-λ system and the potent antitumor activity of IFN-λ in the B16 mouse melanoma model, independent groups confirmed the role of IFN-λ as an antitumoral agent in melanoma and other tumor models. To demonstrate the antitumor activity of IFN-λ, Sato et al. [68] used the mouse melanoma B16F0 and B16F10 and the Colon26 cell lines transfected with IFN-λ2 cDNA. The IFN-λ-transduced B16F0 cells showed an increased activity of caspase 3/7, an induction of p21 and a dephosphoryation of Rb, which triggered a cell cycle arrest and apoptosis. These events, obtained, *in vitro*, were apparently associated with a growth delay, observed *in vivo* after the injection of the B16F0 transduced with IFN-λ. A delay in tumor growth was also observed after the administration of the Colon26 cells transduced with IFN-λ. By using the B16F10 cell line, which represents metastatic mouse melanoma cells, the authors showed that the overexpression of IFN-λ significantly inhibited lung metastasis. In another study, to evaluate the antitumor activity of IFN-λ, Numasaki et al. [69] first transduced the mouse fibrosarcoma cells, MCA2005, with the retroviral vector PA317IL-28 (IFN-λ2). Following the injection of the engineered tumor cells to mice, the authors observed a significant antitumor and antimetastatic effect in mice inoculated with the MCA2005IL-28 in comparison with those injected with the parental tumor cells.

3.2. Investigation of the Antitumor Activities of IFN-λ in the BNL Mouse Model of Hepatocellular Carcinoma (HCC). HCC is the most prevalent type of liver cancer. It is the fifth most common solid tumor and the third leading cause of cancer-related death worldwide. It is also the second most lethal cancer with the five-year survival rate below 9% [70–72]. Treatment options for HCC are limited mainly because of the inefficiency of existing anticancer chemotherapeutic drugs against HCC. Unfortunately, due to a lack of biomarkers and screening for HCC, most patients are diagnosed at advanced stages of the disease and do not meet strict selection criteria for potentially curative surgical tumor resection or orthotopic liver transplantation (OLT) [73–75]. In patients with unresectable HCC and preserved liver function, transarterial chemoembolization (TACE) has been shown to prolong survival. However TACE is rarely curative, and progression-free survival beyond 24 months is not frequent [71, 76]. For patients with advanced disease, systemic chemotherapy is of limited benefit because of the resistance of HCC to existing anticancer drugs and the fact that about 50% of patients with HCC die secondary to liver failure from cirrhosis [77, 78]. HCC occurs most frequently in patients with cirrhosis as a result of chronic HBV (hepatitis B virus) and HCV (hepatitis C virus) infections, and alcohol abuse [72, 79]. Although the link between the cancer and the viral infection is not fully understood yet, there is some suggestion that viral infection interferes with signal transduction and consequently disrupts the normal, controlled growth of cells.

Since IFN-α is used in the clinic for the treatment of chronic HCV and HBV infections, several studies evaluated the effect of IFN treatment on the incidence of HCC [72]. It was previously shown that the systemic administration of high doses and long-term IFN-α into nude mice bearing human HCC with high metastatic potential, following curative resection, inhibited tumor metastatis and recurrence [80]. The majority of clinical studies also concluded that IFN therapy, alone or in combination with ribavirin, decreased the incidence of HCC, particularly in patients with sustained virological response [81–84]. Therefore, IFN alone or, perhaps, in combination with other drugs can be used as a preventive therapy against the development of HCC in HCV- and HBV-infected patients. However, numerous side effects limit the overall tolerability of IFN-α, particularly in patients with cirrhosis [85–87].

In the following part of this section, we describe our findings on the antitumor properties of IFN-λ in the BNL mouse model of HCC. To evaluate the antitumor activities of both IFN-λ and IFN-α, we used a gene therapy approach as previously described [4]. We expressed IFN-λ and IFN-α genes under a strong constitutive promoter in BNL cells and selected stable cell lines, BNL-IFN-λ and BNL-IFN-α, constitutively expressing IFN-λ and IFN-α [45]. Since the constitutive expression of IFN-λ at the tumor site was found to affect the tumorigenicity of B16 melanoma cells *in vivo* [4], we examined whether similar effects of IFN-λ would be displayed in the case of BNL hepatoma. Mice injected with BNL vector or parental BNL cells developed tumors in 4 to 6 weeks, whereas the tumor appearance for BNL-IFN-λ cells was significantly delayed. Similar effects were obtained in mice inoculated with BNL-IFN-α cells. These experiments demonstrated that constitutive expression of IFNs at the tumor site resulted in the delay of tumor growth *in vivo*. Interestingly, we found that IFN-α and IFN-λ exhibited similar antitumor activities [45].

4. Potential Antitumor Mechanisms of IFN-α and IFN-λ

4.1. Antitumor Mechanisms of IFN-α. Despite the antiproliferative effects of IFN-α, it seems that the direct effects on tumor cells may not be the major mechanism by which IFN-α displays its antitumor activity. IFN-α can act indirectly on the tumor by inhibiting angiogenesis which is induced by the tumors and is required to promote their growth and metastasis [88]. In mice bearing human tumors, it was clearly demonstrated that the antitumor activity of IFN-α is associated with the inhibition of tumor angiogenesis in bladder carcinoma [89] and prostate cancer [90]. The involvement of the immune system in the antitumor mechanism of IFN-α was strongly suggested by Gresser et al. [91, 92]. Early studies in tumor models have shown that an intact immune system was essential in IFN-α-induced antitumor activities. The inhibition of Friend leukemia cells (FLC) by IFN-α in mice was shown to depend on the activation of host cells, such as NK cells and macrophages [92]. Both host humoral and cellular immune mechanisms were involved in the continued suppression of Friend erythroleukemia metastases after IFN-α treatment in mice [91]. In addition, effective adaptive immunotherapy was observed in a T-cell lymphoma model, after the injection of tumor-sensitized spleen cells and IFN-α. By using antibodies against different immune cell populations, it has been shown that $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes were the major effectors in the antitumor activities induced by IFN-α [93, 94].

4.2. Antitumor Mechanisms of IFN-λ. Although IFN-α and IFN-λ signal quite similarly (Figure 1), the mechanisms underlying the antitumor activity of IFN-λ may be qualitatively different from IFN-α. As previously described, we initially investigated whether type III IFNs also possessed antitumor activities utilizing a gene therapy approach in the B16 melanoma model. Since secreted IFN-λ did not affect the proliferation rate of B16 melanoma cells *in vitro*, studies in the B16 melanoma model suggested that IFN-λ acted through host mechanisms to elicit its antitumor activity [4]. However, we did not observe a significant long-lasting immunity, implying that there may be a lack of effective adaptive immunity in the mice which rejected the tumor. On the other hand, we noticed a reduction in tumor vascularity in the presence of IFN-λ, suggesting a potential role of IFN-λ in the tumor microenvironment [4]. Since we found that keratinocytes are highly sensitive to IFN-λ and they are known to interact with melanocytes, the cells from which the melanoma originates, we suggested that IFN-λ delivery to the tumor microenvironment may affect the function of the keratinocytes as well as other stroma cells thereby promoting inhibition of tumor growth [4]. NK cells, the major effectors of innate immunity, could also be recruited to the tumor microenvironment and help destroy the tumor cells. Two groups have reported that NK cells played a role in the antitumor mechanisms of IFN-λ. Sato et al. [68] have described the involvement of NK cells in melanoma and colon cancer antitumor responses. They have shown that transient transduction of B16 cells with mouse IFN-λ cDNA enhanced MHC class I and Fas expression, suppressed cell proliferation by inducing increased caspase-3/7 activity, increased $p21^{Waf1/Cip1}$ levels, and dephosphorylated Rb (Ser^{780}) *in vitro* [68]. This meant that IFN-λ was able to induce cell cycle arrest and apoptotic cell death *in vitro*. In addition, they have demonstrated that overexpression of IFN-λ inhibited local and pulmonary metastatic tumor formation *in vivo*. Depletion of NK cells, by injecting an anti-asialo GM1 antibody before tumor cells injection, revealed that NK cells are important in this IFN-λ-mediated tumor growth inhibition *in vivo*, suggesting that IFN-λ activated the innate immune response [68]. Numasaki et al. [69] have also implicated NK cells, polymorphonuclear neutrophils, and $CD8^+$ T cells in the antitumoral activity are induced by IFN-λ in the MCA205 murine fibrosarcoma mouse model. Inoculation of MCA205-IFN-λ cells into mice enhanced IFN-γ production and cytotoxic T-cell activity in the spleen. The antitumor activity of IFN-λ was partially dependent on IFN-γ. In addition, IFN-λ increased the total number of splenic NK cells in severe combined immunodeficiency (SCID) mice, enhanced IL-12-induced IFN-γ

production *in vivo,* and expanded spleen cells in C57BL/6 mice. Furthermore, they reported that IL-12 augmented the IFN-λ-mediated antitumor activity in the presence or absence of IFN-γ. Based on their findings, they suggested that IFN-λ is able to induce both innate and adaptive immune responses to suppress *in vivo* tumor growth [69].

Our recent study in the BNL hepatoma model also revealed that NK cells are implicated in the antitumor activity induced by IFN-λ and probably more potently than IFN-α. However, in contrast to IFN-α, we did not detect any response after *in vitro* treatment of NK cells by IFN-λ, suggesting that IFN-λ may activate other cells, which then mediate NK cell activation [45]. There was also a marked NK cell infiltration in IFN-λ-producing tumors. In addition, IFN-λ and, to a lesser extent, IFN-α enhanced immunocytotoxicity of splenocytes primed with irradiated BNL cells. Splenocyte cytotoxicity against BNL cells was dependent on IL-12 and IFN-γ and mediated by dendritic cells. In contrast to NK cells, isolated from spleen, $CD11c^+$ and $mPDCA^+$ dendritic cells responded directly to IFN-λ, suggesting that the effects of IFN-λ on NK cells are mediated by other IFN-λ-responsive cells, such as DCs [45]. On the other hand, a significant decrease in $CD4^+CD25^+Foxp3^+$ Tregs was observed in mice inoculated with BNL cells secreting IFN-α, whereas the moderate decrease in Tregs observed in mice receiving BNL cells secreting IFN-λ was not statistically significant [45]. Therefore, antitumor mechanisms activated by IFN-α and IFN-λ may differ; IFN-λ increased the number of NK cells at the tumor site whereas IFN-α had a stronger effect on Tregs in the BNL model.

These studies altogether suggest that although IFN-α and IFN-λ signal quite similarly, differences exist in their biological potency, kinetics, and the sets of target cells sensitive to IFN-λ and IFN-α. Therefore, these two types of IFNs may have distinct physiological functions.

5. IFN-λ and IFN-α: Allies in Achieving Higher Antitumor Activities?

Unlike IFN-α, only a small subset of cells are sensitive to IFN-λ, implying that its potential clinical use may be associated with limited side effects. This presumption raises the question whether IFN-λ could be an alternative to IFN-α in cancer therapy. However, despite the severe and numerous side effects inherent to IFN-α treatment [65], we believe that alternative treatment to IFN-α should be weighed first against the real benefits to patients in terms of overall survival and their tumor clearance. We have demonstrated in the BNL hepatoma model that the combination of IFN-λ and IFN-α could achieve a marked antitumor activity in comparison with the use of each IFN alone [62]. The benefits of the combination therapy of IFN-λ and IFN-α have been demonstrated both by using a gene therapy approach and by direct administration of IFNs to the mice bearing the tumors. The mice injected with BNL cells secreting both IFN-λ and IFN-α can completely reject the tumor, in contrast to the mice that only received the BNL-IFN-λ cells or the BNL-IFN-α cells. Furthermore, mice bearing established tumors and treated with exogenous IFN-λ and IFN-α showed a drastic tumor repression. This effect was observed when the IFNs were delivered locally and even at low doses. Therefore, we believe that IFN-λ is not simply acting like IFN-α, with reduced side effects, but can be combined with IFN-α to achieve efficient antitumor activity. Combination of IFN-λ with low doses of IFN-α, which are subtherapeutic but less toxic [67], may improve IFN therapy and benefit cancer patients. Combinational therapy of IFN-λ and IFN-α may achieve ultimate antitumor activity by inducing complementary mechanisms directly on the tumor cells or by indirectly modulating the tumor microenvironment, thereby leading to the stimulation of the immune response against the tumor and the inhibition of tumor angiogenesis. By acting with different intensities on the same targets, IFN-λ and IFN-α may generate a high level of synergy, leading to a potent antitumor activity.

6. Conclusions

Similarly to IFN-α, IFN-λ has been shown to play an important role in cancer and viral disease treatment. Although the two IFNs act through an identical signaling pathway in the cell, the pattern of their activity seems to be different *in vivo*, implying that IFN-λ and IFN-α are not redundant cytokines. By acting on some targets with different intensities, we believe that IFN-λ and IFN-α act in concert to better control tumor development *in vivo*. Therefore, to achieve better treatments for viral diseases or cancers, we believe that the development of a combination therapy rather than the use of each IFN alone will be more beneficial for the patients. The combination of IFNs with other cytokines, growth factors, or their antagonists could also be an important strategy for the improvement of the IFN therapy. Transforming growth factor-beta (TGFβ) which plays a dual role in cancer, mediating tumor-suppresive activities at early stages and prooncogenic activities at later stages of tumor progression [95, 96], could represent one potentially important modulator or mediator of the IFN response. Understanding the potential crosstalks between IFN-α, IFN-λ and other cytokines or growth factors, such as TGFβ, could be rewarding and lead to new preclinical studies in animal models and new clinical trials resulting in better cancer treatments.

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Clinical Study

The Desmoplastic Stroma Plays an Essential Role in the Accumulation and Modulation of Infiltrated Immune Cells in Pancreatic Adenocarcinoma

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Tumor microenvironment is composed of tumor cells, fibroblasts, and infiltrating immune cells, which all work together and create an inflammatory environment favoring tumor progression. The present study aimed to investigate the role of the desmoplastic stroma in pancreatic ductal adenocarcinoma (PDAC) regarding expression of inflammatory factors and infiltration of immune cells and their impact on the clinical outcome. The PDAC tissues examined expressed significantly increased levels of immunomodulatory and chemotactic factors (IL-6, TGFβ, IDO, COX-2, CCL2, and CCL20) and immune cell-specific markers corresponding to macrophages, myeloid, and plasmacytoid dendritic cells (DCs) as compared to controls. Furthermore, short-time survivors had the lowest levels of DC markers. Immunostainings indicated that the different immune cells and inflammatory factors are mainly localized to the desmoplastic stroma. Therapies modulating the inflammatory tumor microenvironment to promote the attraction of DCs and differentiation of monocytes into functional DCs might improve the survival of PDAC patients.

1. Introduction

Many types of tumors have an inflammatory microenvironment comparable to what is found in chronic inflammatory responses, that is, are enriched in inflammatory cells and mediators, transformed tissue, and increased angiogenesis [1]. The inflammation is created by the interplay between tumor cells and the surrounding stroma, for example, cancer associated fibroblasts (CAF), immune cells, and extracellular matrix and gives rise to an environment favoring tumor expansion [2]. The clinical relevance of the microenvironment regarding tumor progression is supported by the correlations seen between poor outcome and CAFs, angiogenesis and the composition and amount of infiltrating inflammatory cells [3].

Pancreatic ductal adenocarcinoma (PDAC) is a common gastrointestinal malignancy characterized by rapid progression, resulting in poor outcome and a 5-year survival rate of less than 5% [4]. Like in most adenocarcinomas, PDAC has a massive fibrotic stoma, that is, desmoplasia [5–7], which contributes to the local inflammatory environment at the tumor site as well as systemically [8]. The microenvironment found in PDAC supports tumor growth, progression, and the recruitment of leukocytes, such as macrophages, dendritic cells (DCs), T cells, and neutrophils [9–11]. Infiltration of these cells has been detected in a variety of cancers [12, 13].

Several studies have reported that blood DCs and tumor infiltrating DCs exhibit phenotypic and functional abnormalities when isolated from tumor bearing animals and patients with PDAC [14–16]. Given their pivotal role in the adaptive immunity and tumor surveillance in healthy individuals, this impairment might contribute to the tumor escape from the immune system [17]. Increased numbers of DCs have been associated with improved outcome in various types of human cancers, and some studies have also pointed out the DC maturation as a prognostic indicator [18]. We have previously observed a correlation between survival time for PDAC patients and the amount and phenotype of blood DCs, which implicate the importance to maintain a functional DC compartment [16, 19]. Different inflammatory mediators, for example, cyclooxygenase-2 (COX-2), IL-1, IL-6, TGFβ and CXCL8, and their receptors are present in the tumor milieu [20–22]. COX-2 is expressed by several solid tumors, including PDAC, and correlates with tumor invasion and clinical outcome [22]. Moreover, COX-2 is believed to have an influence on the DC impairment, and recent findings have provided evidence that the COX-2 metabolite prostaglandin E_2 (PGE_2) is involved in the upregulation of indoleamine 2,3-dioxygenase (IDO) in DCs [23, 24]. IDO expression transforms DCs into tolerogenic cells that activate regulatory T cells (Tregs), which have been shown to exist in several types of cancers [13].

In the present study we found elevated levels of several inflammatory factors, including CCL2, CCL20, TGFβ, IDO, IL-6, and COX-2, in the PDAC tissue. Furthermore, PDAC tissues had significantly elevated levels of infiltrating macrophages, cytotoxic T cells, and DCs. Low levels of MDC, PDC, and mature DC markers were associated with poor prognosis. Treatments that direct the inflammatory tumor microenvironment to attract high levels of DCs could be beneficial for the clinical outcome of the PDAC patients.

2. Material and Method

2.1. Patients and Controls Enrolled in the Study. Tumor tissues were obtained from 30 PDAC patients undergoing Whipple resection at Linköping University Hospital, Sweden. The patients did not receive any neoadjuvant chemo/radiotherapy and the diagnosis was histologically confirmed by two pathologists. The control group consisted of pancreatic tissue from ten individuals, seven individuals deceased from hypothermia and three patients with benign disease (adjacent pancreatic tissue with normal histology was used in the study). The pancreatic tissues were frozen immediately and cryopreserved in liquid nitrogen. Paraffin-embedded tissue sections from patients and controls were obtained from the department of Pathology at Linköping University Hospital, Sweden and the department of Oncology-pathology, Karolinska Institutet, Stockholm, Sweden. The PDACs were staged according to the 1997 International Union against Cancer classification (TNM = Tumor, Node, Metastasis), and the PDAC patients ranged from T1–T4 (T1 ($n = 3$), T2 ($n = 14$), T3 ($n = 12$), and T4 ($n = 1$)), N0 ($n = 6$), N1 ($n = 24$), and M0 ($n = 30$) stage. The study protocol and patient consent documents were approved by the Regional Ethics committee in Linköping, Sweden (Dnr. M38-06).

2.2. RNA Extraction and Quantification with Real-Time PCR. Total RNA was prepared from the samples using Trizol (Invitrogen) according to manufacturer's protocol and cDNA synthesized with SuperScript III Reverse Transcriptase First-Strand cDNA Synthesis kit according to the manufacturer's protocol (Invitrogen). Quantitative PCR was performed with Fast SYBR Green Master Mix (Version 09/2007; Applied Biosystems, Foster City, Calif, USA) on 7900 Fast Real-Time PCR system with 7900 System SDS 2.3 Software (Applied Biosystems) according to the manufacturer's protocol. Specific primers for CCL2, CCL20, IL-6, TGFβ, CD1a, CD1c, CD68, CD163, CD208, CD209, CD303 (CyberGene AB), and COX-2 (Invitrogen) were used. β-actin, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (CyberGene AB) were utilized as housekeeping genes. The primers were designed using Primer Express (Applied Biosystems). Real-time PCRs for the detection of IDO and PD1 were performed using TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's protocol. All reactions were performed in triplicates including nontemplate controls and two endogenous control probes. FAM conjugated, gene-specific assays were Hs00984148_m1 (IDO), Hs00169472_m1 (PD1) and the endogenous controls Hs01003267_m1 (HPRT1), and Hs99999905_m1 (GAPDH). All reactions were performed in triplicates including nontemplate controls. The results were analyzed using the Ct method [25] and the data were presented as the quantitative expression of each gene.

2.3. Immunohistochemical Staining of PDAC and Normal Pancreatic Tissues. Formalin fixed, paraffin-embedded samples of tumor tissue from PDAC patients ($n = 30$) and normal pancreatic tissue ($n = 10$) were cut in 5 μm sections. The sections were then rehydrated and antigen retrieval was performed in a microwave oven for 15 min (350 W) using citrate buffer (pH 6.0). Endogenous peroxidase was minimized by 10 minutes incubation in H_2O_2, and nonspecific binding was avoided by incubating with Background Sniper (Biocare Medical) or 1% bovine serum albumin for 10 min. The samples were immunostained overnight with antibodies (Ab) for CXCL8 (1 : 25, Becton Dickinson), COX-2 (1 : 200, CRM306B, Biocare Medical), S100 (dilution 1 : 1000, Z 0311, Dako, Sweden), CD163 (dilution 1 : 10, ab74604), CD83 (dilution 1 : 50, ab64875), CD8 (dilution 1 : 600, ab4055), IL-1α (1 : 40, ab7632), CCL2 (1 : 1000, ab73680), and CCL20 (1 : 40, ab9829) (Abcam, UK). The sections were then incubated with alkaline phosphatase conjugated anti-mouse or anti-rabbit secondary Ab (Jackson ImmunoResearch) for one hour or by using LSAB2 System-HRP kit (K0675, Dako) containing biotinylated link and streptavidin-conjugated HRP according to the manufacturer's protocol. Alkaline phosphatase was detected by Vulcan fast red chromogen 2 solution (Biocare Medical) according to the manufacture's protocol. HRP was detected

by development in Tris-buffer containing diaminobenzidine tetrahydrochloride (DAB) (Saveen-Werner AB) and 10 μL of 30% H_2O_2. Counter-staining was performed with methyl green solution (0.1 M sodium Acetate buffer, pH 4.2) containing 1% methyl-green (Sigma Aldrich) or hematoxylin.

2.4. Quantification of Inflammatory Factors and Immune Cells in Tumor Tissue. The amount of CD163, CD8, CD83, and S100 immunoreactive cells and the COX-2 ratio between tumor cells and stroma were analyzed using Quantimet 500MC image processing analysis systems linked to a Leica DM LB microscope (Leica Microsystems) supported by Leica QWin software version 3 (Leica Microsystems). CD163, S100, CD83, and CD8 positive cells were manually marked in 20 randomly chosen fields ($\times$40 magnification) using automated standard operation sequences created by QUIPS (Leica Microsystems), an interactive programming system included in the Leica QWin software. The number of immunoreactive cells/μm^2 pancreatic tissue was calculated. To evaluate the COX-2 ratio between tumor cells and stroma, PDAC tissues were immunohistochemically stained with a combination of rabbit anti-human COX-2 and mouse anti human Ki-67 Ab, followed by alkaline phosphatase- and HRP-conjugated secondary Ab and detected as previously described. Ten randomly selected fields were chosen and areas of double positive cell structures (tumor structures) were marked using an automated standard operation sequence created by QUIPS and compared to nonproliferative COX-2 positive stroma.

2.5. Statistics. The statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, La Jolla, Calif, USA). A P-value < 0.05 was considered statistically significant and error bars throughout indicate standard error of the mean (SEM). Nonparametric data was analyzed using the Wilcoxon matched pairs test followed by Mann-Whitney test. Survival curve was analyzed by the Kaplan-Meier survival method, and statistical significance was determined using Log-rank (Mantel-Cox) test and P value < 0.05 was considered statistically significant.

3. Result

3.1. PDAC Microenvironment Contains Elevated Gene and Protein Expression Levels of Inflammatory Factors. We examined the gene expression levels of several inflammatory factors by qPCR in PDAC tissues and compared them to the levels found in pancreatic tissues from the controls. IL-6 ($P = 0.023$), COX-2 ($P < 0.001$), CCL2 ($P = 0.035$), CCL20 ($P < 0.001$), TGFβ ($P = 0.016$), and IDO ($P = 0.003$) gene expression levels were all significantly increased in PDAC tissues compared to controls (Figures 1(a)–1(f)). Gene expression of programmed death-1 receptor (PD1) was not detected in any of the control samples ($n = 10$), but detected in 40% of the tumor samples ($n = 30$) (data not shown).

3.2. Distribution of Inflammatory Factors in Tumor Cells and Stroma in PDAC Tissues. The position of the inflammatory factors detected in the tumor microenvironment was done by immunohistochemistry (IHC). We stained PDAC and normal pancreatic tissues with Saffron and Hematoxylin, known to visualize fibrotic tissues. This staining demonstrated that the tumor cells were surrounded by a massive desmoplastic stroma in PDAC (Figures 2(a) and 2(b)). IL-1α was exclusively located in tumor cells while normal pancreatic tissue stained negative (Figure 3(a)). CCL2 was expressed by fibrotic stroma and Langerhans islets, in both PDAC and healthy pancreatic tissues. CXCL8 and CCL20 were exclusively expressed by PDAC tissue and mainly localized to the stroma cells, but CCL20 was also detected in tumor cells (Figures 3(b)–3(d)). We found COX-2 expression to be restricted to PDAC samples, where several cell types, for example, tumor cells, CAFs, Langerhans Islet cells, and infiltrating immune cells in the tumor expressed this inflammatory mediator with significantly higher expression in the stroma than in tumor nests ($P = 0.028$) (Figures 3(e) and 3(f)). These findings indicate that the stroma, that is, nonneoplastic tissue, constitutes an important contributor to the inflammation seen in the tumor microenvironment in PDAC.

3.3. PDAC Tissue Shows Elevated Gene Expression of Markers Associated with the Expression of Macrophages and Dendritic Cell Subtypes. The presence and activation status of tumor infiltrating immune cells was assessed by qPCR using cell-specific markers unique for DCs, or macrophages. We found significantly increased levels of macrophages as measured by CD163 ($P < 0.001$) (Figure 4(a)) and CD68 ($P < 0.001$) (Figure 4(b)), myeloid DCs as measured by CD1a ($P = 0.032$) and CD1c ($P < 0.001$) (Figures 4(c) and 4(d)), and plasmacytoid DCs as measured by CD303 ($P = 0.007$) (Figure 4(e)). The DCs displayed an activated phenotype with significantly increased CD83 ($P < 0.001$), CD208 ($P = 0.008$), and decreased CD209 ($P < 0.001$) (Figures 4(f)–4(h)). Of note, we cannot rule out that a fraction of the CD209 expression detected is due to other cells than DCs as a small subpopulation of tissue macrophages can express this lectin [26].

3.4. Enhanced Levels of Infiltrating Immune Cells Such As Macrophages, Dendritic Cells, and Cytotoxic T Cells in the PDAC Stroma. We assessed the presence of macrophages, DCs, mature DCs, and cytotoxic T cells in the PDAC microenvironment by immunostaining. Infiltrating CD163 positive macrophages were found in the tumor stroma, and the levels were significantly higher than in the control group ($P < 0.001$) (Figures 5(a) and 5(e)). S100 positive DCs were significantly increased in PDAC compared to normal pancreas ($P = 0.018$). The majority of S100 positive cells in PDAC tissue were located in the fibrous stroma, often in close relation to tumor nests, whereas DCs in healthy pancreas tissues were mainly found in the Langerhans islets (Figures 5(b) and 5(e)). The infiltration of CD83 positive mature DCs varied from low to massive between the different

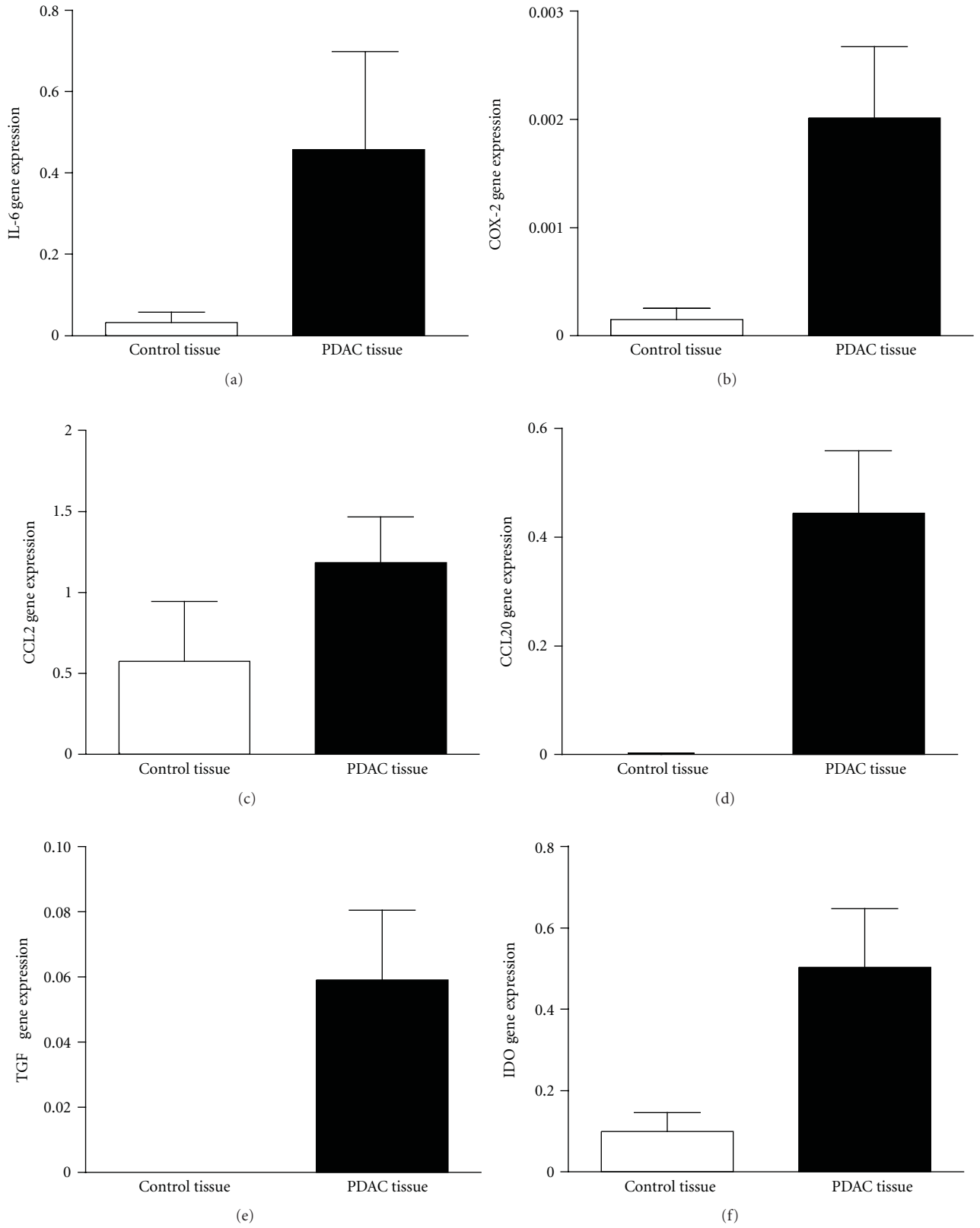
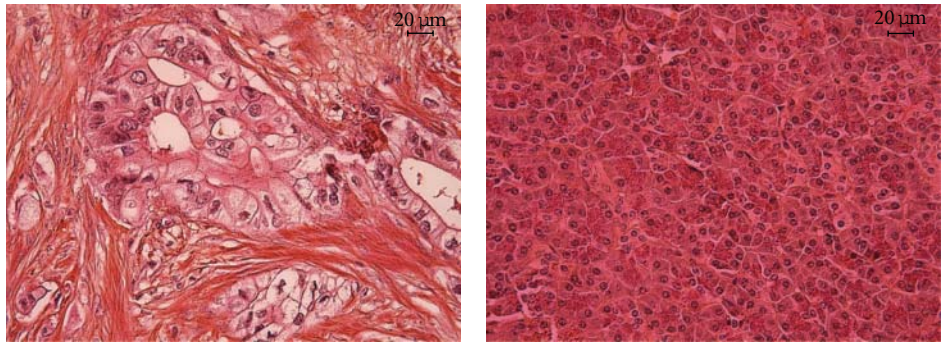


Figure 1: Elevated gene expression levels of inflammatory factors in PDAC tissue. RNA was extracted from PDAC ($n = 30$) and normal pancreatic tissue samples ($n = 10$) and assessed for relative gene expression levels of the inflammatory factors, IL-6 (a), COX-2 (b), CCL2 (c), CCL20 (d) TGFβ (e), and IDO (f). $^{*}P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.001$.



(a) (b)

Figure 2: PDAC tissues contain an extensive fibrotic component. Normal pancreas from individuals deceased from hypothermia, and PDAC tissues were stained using Saffron and Hematoxylin. Photographs show the staining for PDAC (a) and normal pancreas (b), the fibrotic tissue is visualized as orange. Size bar 20 μm.

PDAC patients but showed significantly increased numbers compared to healthy pancreas tissue ($P = 0.004$). The highest extent of CD83 positive dendritic cells in PDAC were found in the fibrous stroma (Figures 5(c) and 5(e)). Cytotoxic CD8+ T cells (CTLs) were not found in healthy pancreas, but PDAC tissues had an infiltration of CD8+ T cells in the fibrous stroma surrounding the tumor nests. The numbers of CD8+ T cells found in PDAC samples were significantly higher than in controls ($P < 0.001$) (Figures 5(d) and 5(e)). To make sure that ischemia induced by the surgical procedure was not influencing the amount of infiltrating immune cells in the tissue samples, we compared Whipple resected pancreatic tissue from patients with cystic lesions; tumor adjacent pancreatic tissue (with normal histology) as well as pancreatic tissues obtained from individuals deceased from hypothermia (data not shown). We did not see any difference in the amount of inflammatory cells or expression levels of inflammatory markers for patients with or without stent (data not shown), which is supported by the literature showing only superficial infiltration of inflammatory cells at the location of nondrug delivery stents [27–29]. To eliminate jaundice as a factor modulating the immune cells in our study, we compared the patients' blood bilirubin levels with the levels of the immune cell markers assessed in this study and found no correlation.

3.5. PDAC Patients with Higher Levels of Dendritic Cells and Macrophages with CD163 Dominating Phenotype Had the Longest Survival Time. The impacts DCs and macrophages have on patient survival were tested by dividing the patients into three groups based on the survival time after tumor resection (short = less than one year ($n = 9$), moderate = between 1 and 2 years ($n = 10$) and long = more than two years ($n = 11$)). The short-time survivors expressed significantly lower gene levels of myeloid DC (CD1c+) and plasmacytoid DC (CD303+) markers as compared to the moderate survivors ($P = 0.017$ (CD1c) and $P < 0.001$ (CD303)). Higher gene expression levels were also observed among the long time survivors, but the difference was not significant (Figures 6(a) and 6(b)). The number of S100 positive DCs in PDAC tissue was higher in the patients surviving more than 2 years compared to patients surviving less than one year, but the difference was not significant ($P = 0.06$) (data not shown). The gene expression levels of the DC activation markers CD208 and CD209 showed higher levels of both tumor infiltrated mature and immature DCs among the moderate ($P = 0.07$ (CD208) and $P = 0.008$ (CD209)) and long-time survivors ($P = 0.012$ (CD208) and $P =$ ns (CD209)) as compared to short-time survivors (Figures 6(c) and 6(d)). The short time survivors were also found to have tumors expressing the lowest levels of the macrophage marker CD163 compared to the moderate survivors ($P = 0.014$), and the same tendency was observed among the long-time survivors but the difference was not significant (Figure 6(e)). The tumor expression levels of CD68, another macrophage marker, indicated the long-time survivors to express the lowest levels of CD68+ macrophages (Figure 6(f)). To further investigate the clinical outcome of the expression of CD68 and CD163 by macrophages, we divided the patients into two groups based on if their main gene expression was CD68 or CD163. Patients with a dominating gene expression of CD163 were presented with significantly better clinical outcome than patients with a CD68 dominating macrophage population ($P = 0.017$) (Figure 6(g)).

4. Discussion

The composition of the tumor microenvironment is essential for the tumor development and will influence the ability of the immune system to mount a defense against the tumor. PDAC tissues contained several types of inflammatory immune cells, that is, macrophages, MDCs, PDCs, and CTLs, besides high levels of inflammatory factors including IL-1α, IL-6, COX-2, TGFβ, CXCL8, CCL2, and CCL20. The inflammatory factors produced by tumor and stroma cells, including immune cells, CAFs, and Langerhans islet cells, create an environment that could support survival and progression of the malignant cells by altering the inflammatory balance in favor of the tumor.

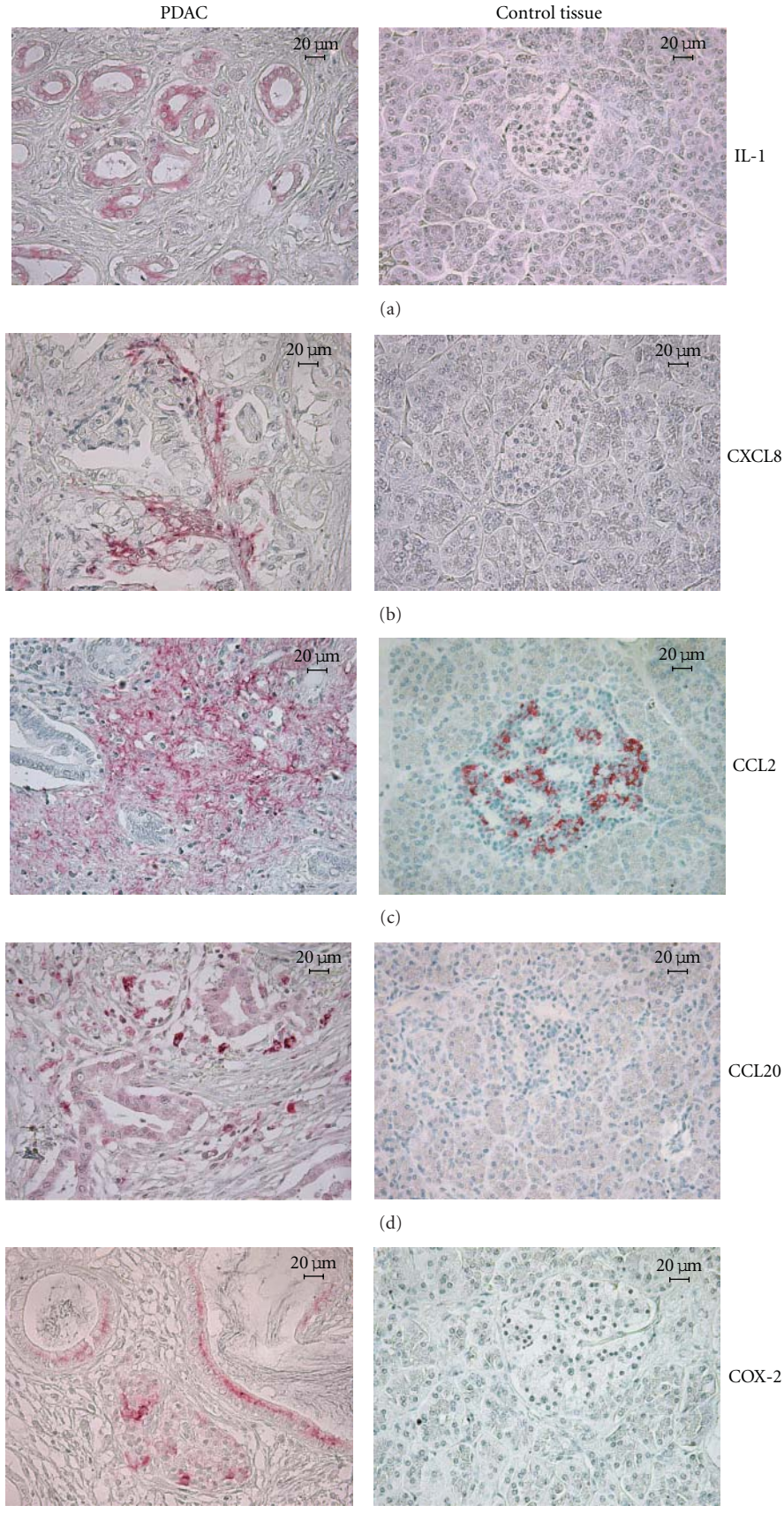
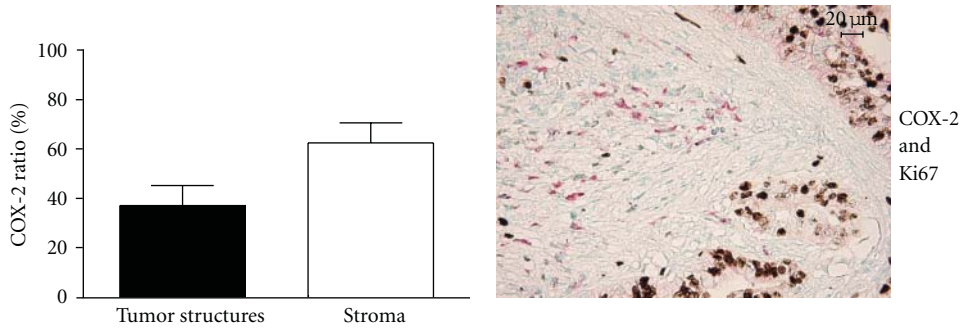


Figure 3: Continued.



(f)

Figure 3: The majority of inflammatory factors in PDAC microenvironment were expressed by the stroma cells. Human PDAC tissue sections were immunostained with anti-COX-2, -CCL20, -CCL2, -CXCL8, and -IL-1α antibodies (abs) followed by alkaline phosphatase-conjugated secondary abs and visualized with fast red chromogen. Photographs show the staining (red) for (a) IL-1α positive tumor structures, (b) CXCL8 positive tumor stroma, (c) CCL2 positive fibrotic cells, (d) CCL20 positive tumor and stromal cells, and (e) COX-2 positive tumor cells and stroma. Size bar 20 μm. (f) To evaluate the ratio between tumor cell and stroma expressed COX-2, PDAC tissue sections were immunohistochemically stained with anti-COX-2 (red) and anti-Ki-67 abs (brown), followed by alkaline phosphatase- and HRP-conjugated secondary abs and detected using fast red chromogen and DAB, respectively. Areas of double positive cells (tumor nests) were compared to nonproliferative COX-2 positive stroma using a computerized image processing analysis system. $^*P < 0.05$.

In healthy human pancreas the Langerhans islets were found to be the main reservoir of DCs and, to our knowledge, this has previously only been reported in mice [30]. An obvious relocation of DCs was seen in the PDAC tissues, where most DCs were located in the fibrotic stroma. The expression of DCs were found to be higher in the tumors than in normal healthy pancreas, but in general the PDAC tumors showed very low levels of both MDCs and PDCs, and a shortage of DCs, mature or immature, was associated with poor clinical outcome. The low levels of DCs and the location in the tumor stroma confirmed previous findings by Dallal et al. [31], but with the use of gene-specific markers we have further extended these findings to include immunomodulatory and chemotactic factors. Furthermore, we were able to show a connection between the DC infiltration and the clinical outcome of the patients.

The chemokines produced by the fibrotic stroma in PDAC tumors, including CXCL8 and CCL20, have previously been shown to initiate the migration of DCs to this site [32]. Moreover, CXCL8 derived from tumor cells have been shown to retain DCs in the tumor resulting in deficient migration to the lymph nodes and also impaired immune response against the cancer [33]. This is also supported by our findings of elevated levels of mature DCs expressing the phenotypic maturation markers CD83 and CD208 in addition to decreased levels of CD209. Nevertheless, patients with low levels of infiltrating CD208 positive DCs had the shortest survival time among the PDAC patients which is in accordance with findings in melanoma [34]. This could indicate an important role for the composition of the inflammatory tumor microenvironment and its ability to retain and mature the DCs.

PDCs are normally found in blood but can also be found at sites of chronic inflammation including cancers [35]. PDAC tumors have been shown to express high levels of CXCL12 and CCL2, which could promote PDC migration into the tumor [36, 37]. This is in accordance with our data showing the presence of PDCs in PDAC tissue and the lack of PDCs in healthy pancreatic tissue.

IDO expressed by DCs or cancer cells have been shown to suppress the immune response to tumors by establishing immunological tolerance [38]. Furthermore, the expression of IDO in ovarian, endometrial, and colon cancer has been correlated to poor clinical outcome [39]. The COX-2 product PGE_2 is known to be an inducer of IDO expression in antigen presenting cells and inhibition of COX-2 expression both in vitro and in vivo reduced the expression of IDO [40]. This is in accordance with our data showing increased expression of both COX-2 and IDO in the PDAC tissues. Consequently, the elevated levels of IDO detected in the PDAC tumors could be a contributing factor to the lack of an efficient immune response against the tumor.

Another factor associated with immune tolerance, TGFβ, expressed by tumor cells and tumor infiltrating DCs has been shown to promote the expansion of natural occurring Tregs (nTregs) [41, 42]. In the present study, PDAC but not healthy pancreatic tissue expressed TGFβ which might contribute to the immune suppression by promoting the expansion of nTregs in the tumor microenvironment and this needs further investigation.

The negative immunoregulatory receptor PD1, expressed by activated T cells, was detected in 40% of the PDAC tumors. The presence of PD1 positive immune cells has been shown to be associated with adverse pathology and poor outcome in patients with renal cell carcinoma [43, 44] and might also be involved in the impaired immune response against PDAC tumors.

Macrophages are derived from progenitors, that is, monocytes existing in the circulation, and are recruited to tissues under the influence of CCL2 [45], which was found elevated in PDAC tissue in the present study. Macrophages may enhance the tumor growth as they secrete VEGF-A,

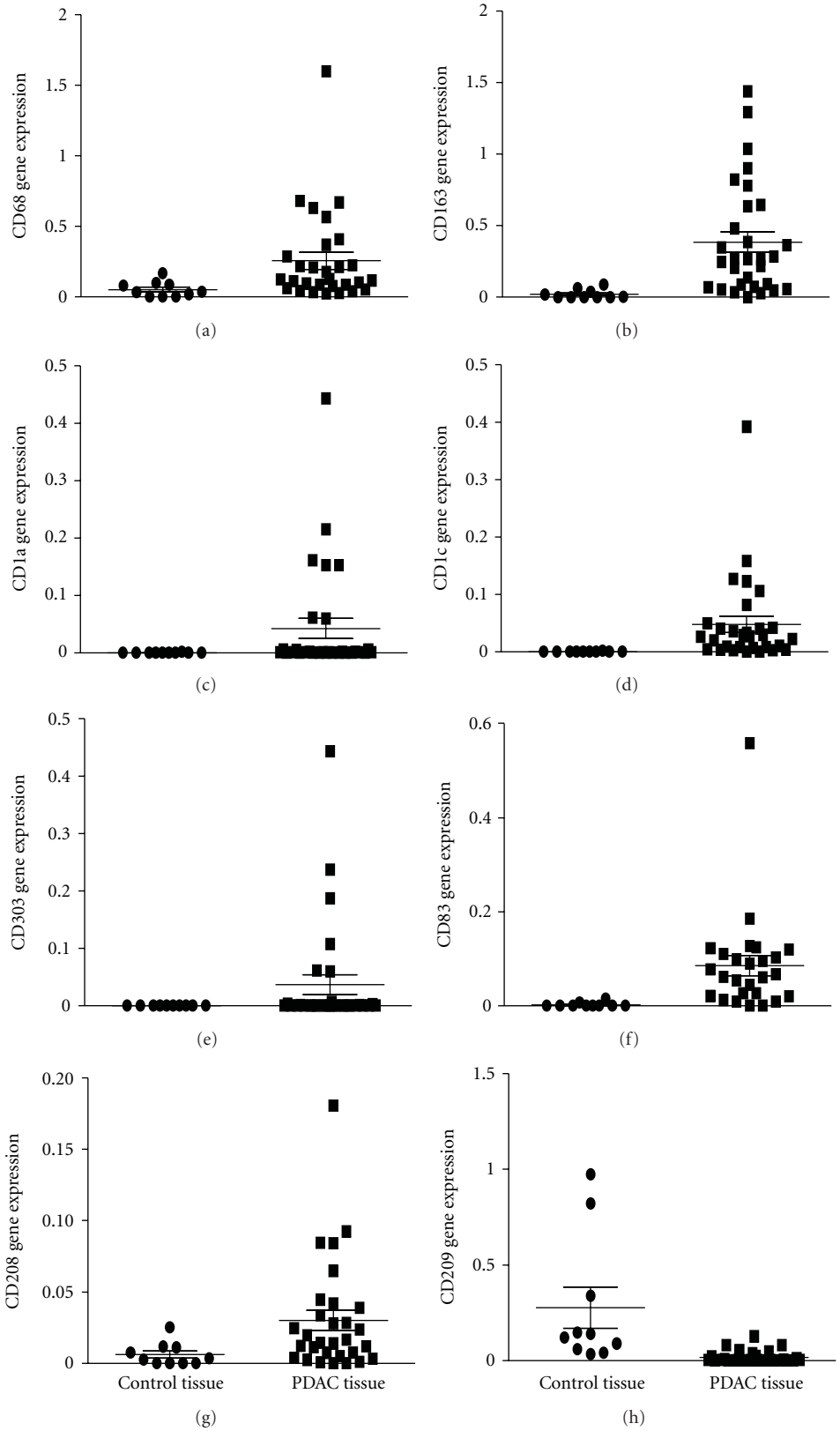


Figure 4: Elevated gene expression levels of markers of infiltrating immune cells in PDAC tissue. RNA was obtained from PDAC ($n = 30$) and normal pancreatic tissue samples ($n = 10$) and assessed for relative gene expression levels of markers expressed by (a and b) macrophages (CD68 and CD163), (c and d) myeloid dendritic cells (CD1a and CD1c), (e) plasmacytoid dendritic cells (CD303), and (f–h) DC maturation/activation status markers (CD83, CD208 and CD209). $^{*}P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.001$.

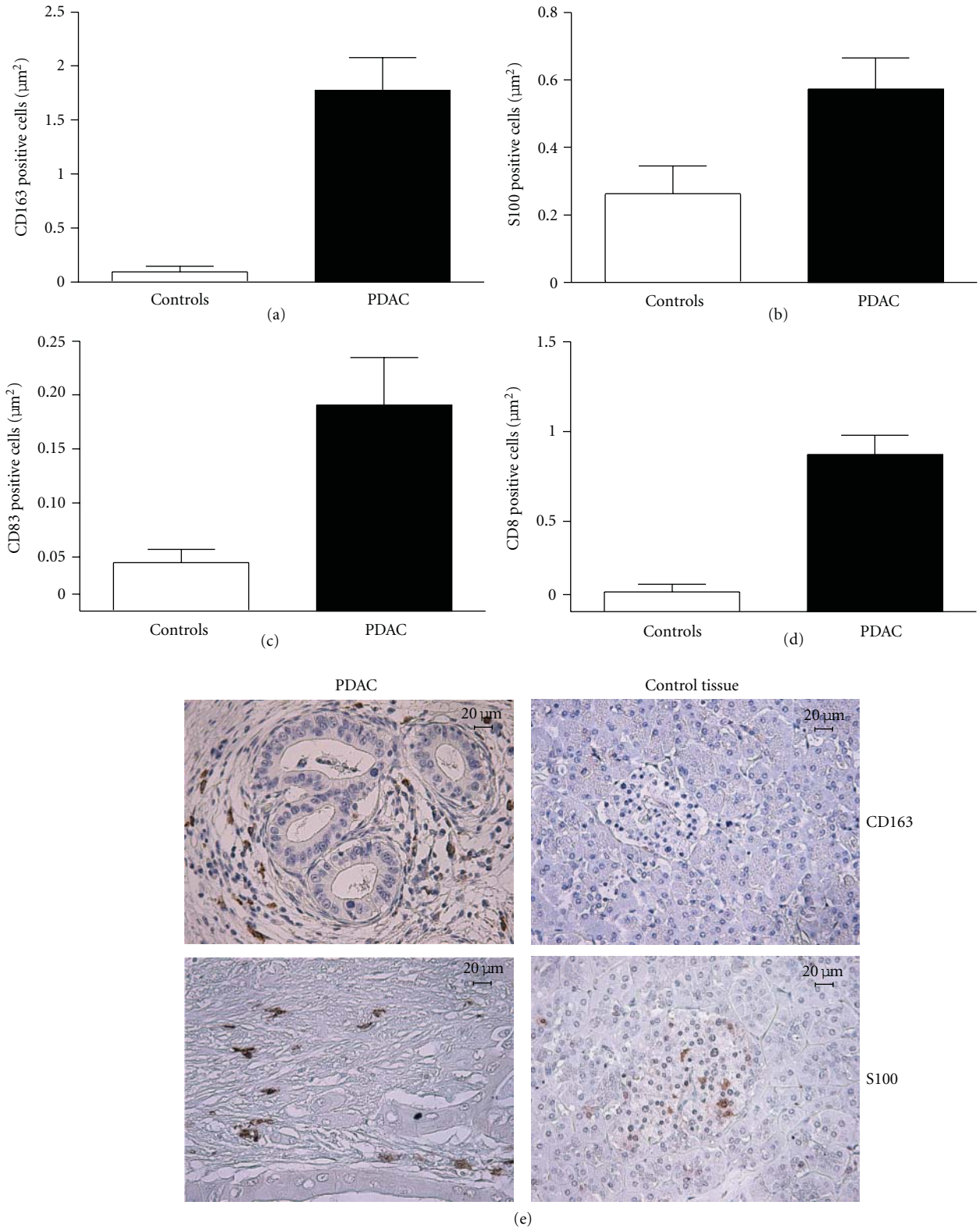


Figure 5: PDAC tissues show infiltration of immune cells such as macrophages, dendritic cells, and CTLs. PDAC and healthy pancreatic tissue samples were immunohistochemically stained with anti CD163, S100, CD83, and CD8 antibodies (abs) followed by biotin-conjugated secondary abs and streptavidin-HRP complex. Peroxidase was detected using DAB chromogen. The amount CD163 (a), S100 (b), CD83 (c), and CD8 (d) positive cells/μm^2 were calculated from 20 randomly selected fields using a computerized image processing analysis system linked to a microscope. (e) The micrographs show expression and location of CD163, S100, CD83, and CD8 positive cells in the PDAC and healthy pancreatic tissues. Size bar 20 μm. $^*P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.001$.

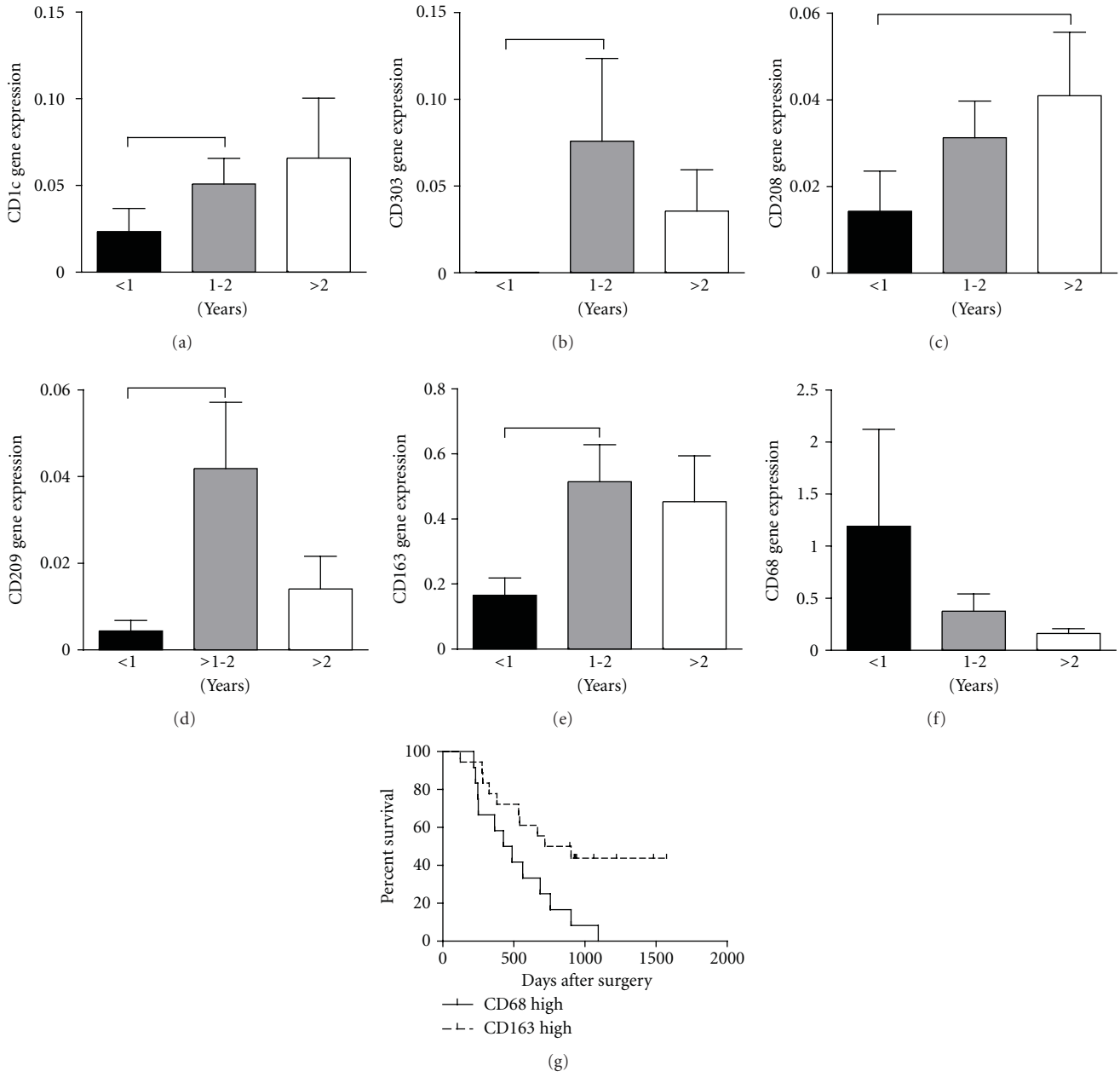


Figure 6: The expression levels of dendritic cell and macrophage markers might predict the PDAC patient survival. The PDAC patients were divided into three groups based on their survival time after tumor resection (less than one year ($n = 9$), between 1 and 2 years ($n = 10$), and more than two years ($n = 11$)). The gene expressions of CD1c (a), CD303 (b), CD208 (c), CD209 (d), CD163 (e), and CD68 (f) were compared between each group. The patients were also divided into two groups based on if the main expression of macrophage markers was CD68 ($n = 12$) or CD163 ($n = 18$) (g). Log-rank (Mantel Cox) test was used for calculation of P value. $^{*}P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.001$.

VEGF-C, and FGF, which are known to contribute to angiogenesis in tumor and also to increase the metastatic potential of tumor cells [9]. When infiltrating the tumor microenvironment, these cells are referred to as tumor-associated macrophages (TAMs). TAMs are important inflammatory cells correlating with tumor progression and bad prognosis in, for example, breast, lung, and cervix cancer {Leek, 2000 #160; Zhang, #157; Pollard, 2004 #151}. Our data suggest that high gene expression levels of CD68 might be associated with poor prognosis, though nonsignificant, while high levels of CD163 were found among the patients with the best clinical outcome, which could point toward the presence of two different types of TAMs with opposite functions. This was supported by our findings pointing to a survival advantage for patients with a CD163 dominating macrophage phenotype. Moreover, the PDAC tumors expressed macrophage markers to a higher extent than DC markers which might at least partly be explained by the increased expression of IL-6

which has been shown by Chomarat et al. [46] to promote the differentiation of monocytes into macrophages at the expense of DCs [46]. We have previously identified IL-1α to be the main tool used by the tumor cells to activate CAFs to produce several inflammatory factors (e.g., IL-6, CCL20, CXCL8, COX-2, and VEGF-A), and this could be one mechanism the tumor cells use to escape elimination by the immune system. Blocking the IL-1 signaling cascade using synthetic IL-1RA (Kineret) drastically reduced the expression of the inflammatory factors in vitro [47], and treatment with IL-1 antagonist might thus have the potential to downregulate the levels of immunomodulatory factors in PDAC tumors.

This study points to the importance of the fibrotic stroma in the production of inflammatory factors and accommodation of immune cells in PDAC tumors. Therapies targeting the desmoplastic stroma and/or inflammatory factors such as IL-6, COX-2, CXCL8, and TGFβ might have the potential to manipulate the tumor microenvironment to benefit attraction of DCs and differentiation of monocytes into functional DCs which could affect the clinical outcome for the PDAC patients.

Conflict of Interests

None of the authors has any potential financial conflict of interest related to this manuscript. Marie Larsson and Anna Spångeus shared last authorship.

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Review Article

The Immune Response to Tumors as a Tool toward Immunotherapy

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Until recently cancer medical therapy was limited to chemotherapy that could not differentiate cancer cells from normal cells. More recently with the remarkable mushroom of immunology, newer tools became available, resulting in the novel possibility to attack cancer with the specificity of the immune system. Herein we will review some of the recent achievement of immunotherapy in such aggressive cancers as melanoma, prostatic cancer, colorectal carcinoma, and hematologic malignancies. Immunotherapy of tumors has developed several techniques: immune cell transfer, vaccines, immunobiological molecules such as monoclonal antibodies that improve the immune responses to tumors. This can be achieved by blocking pathways limiting the immune response, such as CTLA-4 or Tregs. Immunotherapy may also use cytokines especially proinflammatory cytokines to enhance the activity of cytotoxic T cells (CTLs) derived from tumor infiltrating lymphocytes (TILs). The role of newly discovered cytokines remains to be investigated. Alternatively, an other mechanism consists in enhancing the expression of TAAs on tumor cells. Finally, monoclonal antibodies may be used to target oncogenes.

1. Different Antigenicity of Tumors

An important role of the immune system is to identify and eliminate tumors. Transformed cells of tumors express antigens that are not found on normal cells; these antigens are called tumor-associated antigens (TAAs). The immune system recognized these antigens as not self and mounts an immune response against tumor cells. However, tumors develop several mechanisms to escape immune recognition. For instance, when T cells interact with tumors, they may deliver several potential inhibitory signals, including lack of proper costimulatory activity by tumor cells and induction of immunosuppressive Tregs [1, 2].

In the recent years, specific antigenic characterization has permitted us to study an increasing number of tumors, in particular regarding their ability to escape from immune response and to downmodulate TAA expression and secreting inhibitory molecules. This has resulted in the identification of tumors that elicit different immune responses: (1) strong immunogenic tumors, such as melanoma and renal cell carcinoma, (2) the majority of tumors, however, are poorly immunogenic tumors: these include, for instance, colorectal cancer, hepatocellular carcinoma, pancreatic carcinoma, prostate carcinoma, lymphomas and leukaemias, and others [3, 4].

The tumor-associated antigens (TAAs) expressed by tumors have several sources.

(a) Some are derived from oncogenic viruses like human papillomavirus, which causes cervical cancer [5]. The HPV oncoproteins E6 and E7 have crucial roles in various steps of carcinogenesis, inducing degradation of p53 and destabilization of pRb. Several clinical trials show that recombinant HPV vaccines are safe and effective in preventing persistent infection of HPV and associated anogenital lesions. Thus, prophylactic HPV vaccination may be an ideal preventive method for other HPV-associated cancers. Therefore, vaccine against papillomavirus may be considered a very effective antitumor agent [6–8].

(b) Other TAAs are cellular proteins usually present in the human body that are overexpressed or aberrantly expressed in tumor cells; furthermore, others TAAs are also products of mutated genes.

(c) In addition, TAAs may also be the products of oncogenes or mutated oncosoppressors.

The most useful response of the immune system against tumors is to kill the abnormal cells using CTLs, which abound among TILs [9, 10]. TAAs are presented on MHC class I molecules. This allows CTLs to recognize the tumor cell as abnormal. NK cells also kill tumor cells by cytotoxicity, especially if the tumor cells have fewer MHC class I molecules on their surface than normal; this being a common phenomenon in tumors.

Upon activation, CTLs express on their surface the death activator designated Fas ligand (FasL) and the engagement of Fas/FasL pathway lead to mediated apoptosis of cancer cells [11, 12].

Despite the activity of the immune system, clearly, tumors may evade the immune system and become clinically evident. Tumor cells often have a reduced number of MHC class I molecules on their surface, thus avoiding detection by killer T cells.

An important challenge in cancer immunotherapy is the identification of effective strategies for enhancing its clinical efficacy. One approach is based on adjuvants, capable of breaking tolerance against TAAs. Interferons-alpha (IFN-alpha) are pleiotropic cytokines belonging to type I IFNs, extensively used in the treatment of patients with some types of cancer and viral disease. IFN-alpha can increase the expression of surface antigens enhancing the immune response, acting as an effective adjuvant in cancer immunotherapy [13, 14]. In melanoma it has been demonstrated that IFN-alpha increases the accumulation of gp100-specific, IFN-gamma-secreting CD8+ T cells in the tumor, demonstrating its efficacy as an adjuvant for peptide vaccination and giving insight into its mechanism of action. This provides a rationale for clinical trials in which vaccination is combined with IFN-alpha therapy for melanoma [15]. In addition, IFN-alpha can promote the differentiation and activity of host immune cells. Notably, a special interest is currently focused on the use of dendritic cells (DCs) generated in the presence of IFN-alpha (IFN-DC) for the preparation of anticancer vaccines. An additional approach for enhancing the response to immunotherapy relies on its combination with chemotherapy [16].

Here we will briefly discuss the immunobiology of tumors. Because the topic is too vast for this paper, we will discuss two tumors: melanoma as an example of strong immunogenic tumor and colorectal cancer as an example for poorly immunogenic tumors.

1.1. A Strong Immunogenic Tumor: Melanoma. Malignant melanoma is one of the most aggressive malignancies in human and is responsible for almost 60% of lethal skin tumors. Therapy with IFN-alpha 2b, the only agent approved in the USA for adjuvant use in high-risk melanoma patients, has not shown consistent overall survival benefit in randomized trials and is associated with considerable toxicity. Melanoma is one of the first tumors that have been associated to the presence of local cellular inflammation. The description of a lymphocytic infiltration of primary cutaneous melanoma confirmed Virchow's suggestion of a direct connection between inflammation and cancer. The past 30 years have accumulated considerable evidence that many tumors elicit a significant immune response, and a more favourable prognosis is correlated with the levels of TILs [17]. Nevertheless, although tumor microenvironment TILs include tumor-reactive T cells, melanoma can escape the immune system and continue to grow and metastasize [1]. Studying these mechanisms of immune escape of the tumor will improve the strategies to overcome obstacles to successful immunotherapy of tumors.

Melanoma is characterized by the expression of several TAAs, which can be recognized by T cells, resulting in a strong immunological response to the tumor. These TAAs include gp100, Melan-A/Mart-1, tyrosinase, MAGE-A1, and NY-ESO [1, 18].

Recent data have demonstrated that combined therapeutical approach with chemotherapy and cancer vaccines may have positive effects in the treatment of advanced and metastatic tumors. Chemotherapy, alone or with the association of cancer vaccine, can improve the expression of TAAs and induce enhancement of the cancer-reactive CD8+ cytotoxic T cells (CTLs) [16]. Specific topoisomerase inhibitors can augment melanoma antigens production, suggesting that a combination of chemotherapy and immunotherapy may be of potential value in the treatment of otherwise insensitive cancers [19].

Moreover, recent data have demonstrated the implication of Tregs in the pathogenesis and in the progression of tumors. Tregs mediate their immunosuppressive action also by the expression of the negative costimulatory receptor CTLA-4.

Furthermore, in the last years, the identification of, somatic mutations in the gene encoding the serine-threonine protein kinase B-RAF (BRAF) in the majority of melanomas has resulted in an opportunity to test oncogene-targeted therapy for this disease. Patients with advanced metastatic melanoma have been treated with PLX4032 (Plexxikon; RG7204, Roche Pharmaceuticals), a potent inhibitor of BRAF with the V600E mutation; this treatment resulted in complete or partial tumor regression in the majority of patients [20, 21].

Melanomas share initiating genetic alterations such as oncogenic mutations in BRAF and NRAS and often show recurrent patterns of chromosomal aberrations. Alteration of cell cycle proteins (e.g., cyclin D1, pRb, and p16) has a role in transformation and progression in melanocytic tumors. Higher expression of PAR-1 (protease-activated receptor-1) is seen in melanoma cell lines and tissue specimens [22]. Upregulation of PAR-1 mediates high levels of Cx-43 (gapjunctional intracellular communication molecule connexin) expression. This molecule is involved in tumor cell diapedesis and attachment to endothelial cells [23]. Protein kinase C (PKC) mediates signals for cell growth and is a target of tumor-promoting phorbol esters in malignant transformation [24]. Downregulation of E-cadherin and upregulation of N-cadherin may be seen in melanoma cells. Such shift of cadherin profile may have a role in uncontrolled proliferation, invasion, and migration. Other studies demonstrated the association of vascular endothelial growth

factor (VEGF) and VEGF-receptor family with progression and melanoma metastasis [22].

Effective cancer immunotherapy is dependent on the presence of large number of antitumor lymphocytes with appropriate homing and effector functions that enable them to seek out and destroy cancer cells *in vivo*. Adoptive cell therapy (ACT) refers to an immunotherapy approach in which antitumor lymphocytes are identified and grown *ex vivo* and then infused into the cancer patients, often along with vaccines or growth factors that can augment the *in vivo* impact of the transferred cells. ACT with autologous tumor infiltrating may mediate durable complete responses in patients with metastatic melanoma [25].

1.2. A Poorly Immunogenic Tumor: Colorectal Cancer (CRC). Colorectal cancer (CRC) is one of the leading causes of death in the Western world. Immunotherapy could play a crucial role in patients with advanced disease at presentation permitting tumor regression or possibly clearance. Despite advances in research and treatment modalities, CRC still accounts for around half a million deaths yearly worldwide. Traditional and even newer pharmaceutical therapeutic regimens are limited in terms of tolerance, efficacy, and cross-resistance. Additional non-cross-resistant therapies with nonoverlapping toxicities are needed to improve the outcome for patients with CRC. Cancer vaccines, designed to activate immune effectors (T cells and antibodies) to prevent recurrence or treat advanced cancers, have now demonstrated clinical benefit [26].

Bonertz et al. have found that in CRC Tregs T-cells response is addressed against a limited repertoire of TAAs, which include p53, carcinoembryonic antigen (CEA), Her2/neu, and heparanase pp1 [27].

Colorectal tumor cells frequently express CEA which correlates with the state of the tumor, augmenting its expression in advanced phases. CEA is considered a clinical marker of this tumor, with utility in the diagnosis, prognosis and followup of the disease [28, 29]. Some authors have used anti-CEA antibodies tagged with radioactive Yttrium-90 [30] against CEA-expressing metastatic malignances or combined with antivascular antigens, like combretastatin and bevacizumab (anti-VEGF), or with gemcitabine [31–33]. Moreover, in recent years it has been shown that CRC can express other antigens, such as extracellular surface marker CD55 [34] and the oncofetal antigen 5T4. This latter is a surface glycoprotein expressed on a variety of human adenocarcinomas, including CRC, and plays an important role in tumor progression and metastasis. The expression patterns and functional role in the metastatic process suggest that 5T4 is a good target for vaccine development. A modified vaccine virus Ankara (MVA) encoding human 5T4 (designated TroVax) demonstrated therapeutic effects in murine tumor models and human T cells recognized 5T4 epitopes in an HLA-restricted manner. TroVax vaccine has been evaluated in clinical trials targeting patients with colorectal cancer of advanced stage (IV stage), renal cell carcinoma, and hormone refractory prostate cancer [35]. Results from clinical trials on metastatic colorectal cancer demonstrate that MVA-5T4 is safe and immunogenic as a monotherapy and in combination with standard-of-care therapies. MVA-5T4 induced potent and sustained immune responses in approximately 95% of tested patients. With its minimal side effects and ability to produce immune responses, MVA-5T4 is a promising addition to cancer therapy [36]. Moreover, preliminary results showed significant associations between 5T4-antibody responses and overall survival in patients with CRC. The 5T4-specific antibodies were present at higher levels in cancer patients compared with healthy donors and increased significantly after treatment with MVA-5T4 [37].

Furthermore, CRC can express oncogenes; in particular, KRAS mutations occur in almost 40% of CRC patients. KRAS is a cellular signalling effector downstream from the EGF/EGFR pathway. KRAS mutations are common in colorectal, ovarian, and lung adenocarcinomas. There have been recent attempts to quantify KRAS mutation and predict responses to treatment using an EGFR inhibitors (cetuximab) [38].

In addition, studying on TILs has permitted us to differentiate between the immune cellular populations: Tregs and Th17 cells are involved in the pathogenesis and the progression and proliferation of CRC malignant cells. In particular Tregs and Th17 cells are correlated with a poor prognosis of CRC and with advanced tumors [39]. According to their immune inhibitory function, Tregs depletion results in stronger TAA-specific immune response [27].

2. Biology of the Immune Response to Tumors

2.1. Immune Pathways That Can Potentially Limit Tumor Expansion

2.1.1. Mechanisms of Action in Tumor Vaccines: DCs, CTLs, and Humoral Response. The discovery of high number of tumor-infiltrating lymphocytes (TILs) with skewed tumor-specific TCR expression has promoted the development of both adoptive immunotherapy with transfer of TAA-primed TILs into patients and vaccine-based antitumor therapy [1, 40]. CTLs mediate tumor destruction by the release of perforin [41] and granzymes or by the activation of the Fas-/FasL- mediated apoptosis [42].

Therapeutic tumor vaccines have two main objectives: priming Ag-specific T cells and reprogramming memory T-cells (i.e., a transformation from one type of immunity to another, e.g., regulatory to cytotoxic). Dendritic cells are essential in the generation of immune responses, and as such represent targets and vectors for vaccination [43]. The main goal of tumor vaccine therapy is the production of mature dendritic cells (DCs, the most specialized APCs) able to stimulate an antigen-specific T-cells response *in vivo* [44]. In classical protocol DCs are activated and loaded with TAAs or transfected with RNA-encoding tumoral epitopes and then transferred to tumor-bearing hosts [45, 46]. Notably, most antigens expressed on tumor cells are self antigens and may result in poor antigenicity due to negative selection of high-avidity autoreactive T-cell subsets; moreover antigens expression depend on the proteolytic processing by immunoproteasomes and differential binding to allelic MHC variants

leading to the hiding of "cryptic" specific epitopes [47]. Therefore the antigen presentation may be different in different cells, and a selection of proper antigenic peptides may be useful to mediate efficient killing of cancer cells [47].

2.2. Immunological Pathways That Can Limit the Immune Responses

2.2.1. The Role of Tregs. The heterogeneity of CD4 cells has been described in the past [48], but only recently a CD4 T-cell subpopulation with regulatory function (Tregs) has been characterized functionally. Regulatory T cells (Tregs) represent about 5–10% of peripheral CD4+ T cells; they are characterized by the ability to suppress T-cell responses. If this function is impaired, the host will be exposed to dysfunctions in self-tolerance. Several diseases have been linked to defective Treg activity including type I diabetes, allergy, and other autoimmune diseases [39, 49–51].

In tumors, several studies suggested a direct correlation between adverse prognosis and presence of Tregs in peripheral blood as well in TILs and in draining lymph nodes of different tumors [52].

Tregs express a number of chemokine receptors such as CCR2, CCR4, CCR5, CCR7, CCR8, and CXCR4 and are able to migrate in response to a variety of chemokines such as CCL22, CCL17, CCL1, and CCL4 [53–55]. Tregs may be recruits to the tumor site by the chemokine CCL22 produced by the tumor cells and tumor-associated macrophages (TAMs). Tregs accumulated via CCL2-CCR4 recognize tumor-associated immunogenic self-antigens (self-Ags) and proliferate [52]. Moreover, a recent study on breast cancer showed that the hypoxia environment drives the Tregs recruitment through both CXCL12 production by tumor cells and hypoxia-induced CXCR4 expression in Tregs [56].

Moreover, Tregs selectively recruited within the tumor site will be activated by mature DCs likely through TAA; therefore, Tregs induce T-cell suppressions in an antigen-selective manner [52]. Indeed it is also clear that vaccination with some of these epitopes, administered with or without an adjuvant or presented by *ex vivo* cultured antigen-presenting cells (APCs), can induce humoral and CTL antitumor responses in some cases [57].

It has been reported that immunosuppressive factors produced in the growing tumor environment, such as TGF-beta, IL-6, and IL-10, created an immunosuppressive environment. Therefore, both the tumor cells (by their expression of tumor antigens and production of these factors) and/or the TAMs may act via promoting the antigen-activated T cells to differentiate and proliferate into Treg cells [58].

Tregs suppression may therefore impair cancer immunotherapies [52]. Therefore a clear understanding of the mechanisms of action by Tregs in tumor immunity is needed to establish a useful tumor vaccine or immunotherapy [59, 60]. Tregs are highly specific for antigens, suggesting that they exert T-cell suppression in an antigen-selective manner [27].

Recent data, confirming the high presence of Tregs within TILs in the tumor site and in the tumor-draining lymph nodes, however, has demonstrated that regulatory T cells in TILs donot originate by conversion of T-conventional cells (T-conv). Tregs arise from different populations with unique TCR repertoires. Enrichment of Tregs within TILs most likely, therefore, reflects differences in the way that Treg and T-conv cells are influenced by the tumor microenvironment. Elucidating the nature of these influences may indicate how the balance between tumor-infiltrating Treg and T-conv cells can be manipulated for therapeutic purposes [61].

2.2.2. The Role of CTLA-4 and PD-1. T-cell activation and inhibition are regulated by signalling of several molecules including CD28 that provides costimulation, CTLA-4 (CD152) that binds to the same ligands as CD28, but has more affinity and delivers an inhibitory signal, and programmed death-1 (PD-1) that may be involved in tumor evasion. All these molecules have a potential role in immunotherapy [39].

Remarkably, CTLA-4 has more affinity than CD28 for its ligands and can trigger T-cell anergy. CTLA-4 delivers inhibitory signals to T cells blocking their effector functions through different mechanisms including diminishing of TCR signalling, blocking cell cycle progression, and reducing IL-2 production [39].

Also PD-1 seems to be involved in immune evasion, and its expression is reported in melanoma TILs contributing to their impaired antitumor responses [62].

2.2.3. The Role of Cytokines in Regulation of Tumor Antigens. Tumors can mediate their ability to escape immune recognition also secreting immunosuppressive cytokines, such as IL-10 and TGF-beta [63]. Furthermore Tregs can downmodulate immune response by cytokine secretion; these include IL-10, TGF-beta, and the discovered novel IL-35 [64–68]. IL-35 has been shown to be constitutively expressed by regulatory T cells and contributes to their suppressive activity. IL-35 is an important mediator inducing CD4+CD25+ T-cell proliferation and IL-10 production [69].

Recent data have demonstrated also a relation between cytokines and vitamins. In particular, vitamins A, D, and E modulate Treg function and IL-10 and TGF-beta production, involving the immune response mechanisms [70].

Moreover, in addition to the immune cells, also tumors can directly secrete immunosuppressive cytokines, further permitting them to evade the immune response. For example melanoma secretes oncostatin M (OSM), which transmits its signal via the gp130 cell surface receptor, resulting in the selective downmodulation of the melanocyte lineage antigens: Melan-A/MART-1, gp100, tyrosinase, tyrosinase-related proteins 1 and 2, and the M isoform of microphthalmia transcription factor [71]. On the other side it is important to underline that TAAs expression can be modulated in both directions. IFN-beta is an additional stimulus to TAAs expression in melanoma, including Melan-A/MART-1, gp100, and MAGE-A1, permitting an improve of immune response to melanoma cells [1, 18].

2.2.4. The Role of Heat Shock Protein 90 (HSP90). In recent years some data have revealed that the molecular chaperone Heat Shock protein 90 (HSP90) is involved in several condition, including cancer. Hsp90 regulates the trafficking

of proteins in the cell, under stressful conditions, stabilizes its client proteins, and provides protection to the cell against cellular stressors such as in cancer cells. Through its role in regulating the conformation, stability, and function of several key, oncogenic client proteins, HSP90 contributes in maintaining malignant transformation and in increasing the survival, growth, and invasive potential of cancer cells.

HSP90-inhibitors, such as geldanamycin and its analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG, tanespimycin), determine suppression of MAPK pathway in malignant cells and may become new anticancer agents [72, 73]. Moreover, Banerji et al. have shown a correlation between oncogenic BRAF and NRAS mutations, frequently associated with malignant melanoma, and the HSP90. In fact NRAS mutations are stabilized by the molecular chaperone HSP90 and they are depleted by the HSP90 inhibitor 17-AAG [74]. In addition inhibitors may also upregulate TAAs [75].

3. Clinical Approach to Immunotherapy of Cancer

The increased understanding of the mechanisms of immunoregulation has suggested new strategies to design more effective cancer immunotherapies.

3.1. Vaccines: Prostate Cancer and Melanoma. Cancer vaccination is a kind of immunotherapy that relies on specific priming of the immune system in order to stimulate principally adaptive immunity against vaccine component, in contrast to nonspecific immunotherapy where the administered agent tries to enhance the innate immunity (e.g., Bacille Calmette Guérin). Early attempts to develop effective cancer vaccines had limited success due to the failure to identificate suitable target antigens, to mitigate the immunosuppressive environment and generate an effective immune response [76]. However, an improvement in our understanding of the immune system and tumor immunity, in particular, has facilitated the development of more promising vaccine strategies [77, 78].

Different vaccination strategies have been investigated including the use of whole-tumor cells or lysates, dendritic cells, peptide-base approach, recombinant proteins, and viral and DNA delivery vectors. Since antigens are poorly immunogenic by themselves, vaccines generally require the inclusion of potent immunoadjuvants to induce antitumor responses and a delivery system to effectively present the antigen to the immune system [77].

Sipuleucel-T represents the first cancer vaccine approved by the US Food and Drug administration for the treatment of metastatic hormone-refractory prostate cancer. Sipuleucel-T consists of autologous peripheral-blood mononuclear cells including antigen-presenting cells (APCs) that have been activated *ex vivo* with a recombinant fusion protein (PA2024) which contains prostatic acid phosphatase fused to granulocyte-macrophage colony-stimulating factor [79]. In a double-blind, placebo-controlled, multicenter phase III trial, patients with metastatic castration-resistant prostate cancer who received Sipuleucel-T had a prolonged overall survival (median survival 25.8 months in the sipuleucel-T arm versus 21.7 in the placebo group) showing a relative reduction of 22% in the risk of death as compared with placebo arm; also the rate of 3-year survival was increased for patients receiving Sipuleucel-T (31.7%) as compared with those receiving placebo (23%). In particular, patient in the Sipuleucel-T group who had an antibody titer of more than 400 against PA2024 or prostatic acid phosphatase at any time after baseline lived longer than did those who had an antibody titer of 400 or less ($P < 0.001$ and $P = 0.08$, resp.). Adverse events that were more frequently reported in the Sipuleucel-T group included chills, fever, and headache [79].

Several vaccines against melanoma antigens were tested in early clinical trials demonstrating a clinical benefit, but when tested in prospective randomized trials for advance melanoma, they failed to improve progression-free or overall survival compared with chemotherapy. The first evidence of clinical benefit of vaccination for patients with metastatic melanoma came from a prospective randomized phase III trial, conducted with stage IV or locally advanced stage III cutaneous melanoma, HLA A0201+ patients, without brain metastases who received high-dose IL-2 (720.000 IU/kg/dose) as the control group and a gp100 peptide containing a modified 209-217 (210M) epitope + montanide ISA followed by high-dose IL-2 as the experimental arm [80]. The modified g209–217 peptide (referred to as g209-2M) presents a methionine replacing the natural threonine at position 2; it bounds to the HLA-A2 molecule with greater affinity than the unmodified peptide, and it was shown to have an increased ability to generate melanoma-reactive CTLs. Response rate was significantly improved in the experimental arm as compared with control group (22.1% versus 9.7% ($P = 0.0223$), and also progression-free survival favoured the gp100-immunized patients compared to those treated with IL2 alone (2.9 months versus 1.6 months, $P = 0.01$). Overall survival was longer in the experimental group, but the difference was not significant (17.6 versus 12.8 months, $P = 0.0964$).

Other vaccines containing multiple tumor-associated antigens including MAGE proteins, MART-1/MelanA, and gp100 were tested in a phase I/II trial in patients with advanced melanoma with evidence of clinical activity and durable responses [81]. Also vaccines containing dendritic cells pulsed with melanoma-associated antigens or autologous lysates [82], or electroporated with mRNA encoding CD40 ligand, constitutively active toll-like receptor 4, and CD70, are under investigations in metastatic melanoma patients [83].

A vaccine containing a tumor-associated antigen such as MAGE-A3 was also tested in a phase II study for patients with non-small-cell lung cancer after complete resection with improvement in disease-free and overall survival; on the basis of these results, a phase III study with this vaccine was initiated in 2007 and is currently ongoing [84].

Other vaccines produced promising phase III data such as vitespen, an autologous adjuvant vaccine for patients at high risk of recurrence after nephrectomy for renal cell carcinoma [85] and Biovaxid, an idiotype vaccine for patients with follicular lymphoma in first complete remissions [86].

Human telomerase reverse transcriptase (hTERT), the rate-limiting subunit of the telomerase complex, is another attractive target for cancer vaccination since telomerase is highly expressed in almost all cancer forms, while the expression in normal tissues is restricted. Phase I/II trials in advanced pancreatic and pulmonary cancer patients after vaccination with GV1001, a 16-aminoacids-peptide of hTERT sequence, have demonstrated some specific and durable T-cell responses, associated to a prolonged survival, without clinically important toxicity [87, 88].

3.2. Biological Drugs and Their Combination with Cancer Chemotherapy. In contrast to conventional chemotherapy, immunotherapy of tumors has raised the hope of a more specific therapeutical approach in oncology. In fact, immunotherapy, targeting TAAs, has permitted to use novel more specific molecules in cancer therapy.

As discussed above, biological therapies can also stimulate the immune response against cancer. In addition, as we will see, biological therapy can interfere with tumor blood vessel formation therefore blocking its ability to develop.

In some conditions, biological agents may also be administered together with chemotherapy in order to prevent cancer cells from repairing the DNA damage induced by chemotherapy itself. Biological agents can be grouped in two main classes; both these classes have an increasing number of drugs of potential interest and a complete review of them will require a volume and it is beyond the scope of this paper. We therefore will mention those that appear more promising, having in mind that, by the time our paper will appear, several new products will be introduced in the clinical practice.

3.3. Monoclonal Antibodies (mAbs). Ideal drugs would be antibodies against specific antigens on cancer cells that are not cross-reactive with those on normal tissues. mAbs achieve their therapeutic effect through various mechanisms. They can have direct effects in inducing apoptosis or programmed cell death; they can block growth factor receptors, effectively arresting proliferation of tumor cells; they can bring about antiidiotype antibody formation enhancing the patient's immune response [89, 90].

mAbs can be associated to other substance such as a chemotherapy drug, radioactive particle, or a toxin in order to selectively deliver them to a specific cancer cell.

The first monoclonal antibody to receive FDA approval was rituximab, an antibody directed to the CD20 antigen [89, 90]. CD20 is a transmembrane protein whose intracellular portion contains phosphorylation sequences for protein kinase C, calmodulin, and casein kinase 2. Rituximab is active against B-cell lymphoproliferative diseases [89] that are the large majority of lymphoproliferative diseases [91]. When rituximab cross-links CD20 antigen, an increase in intracellular calcium is observed. This increase appears to activate the SER family of tyrosine kinases, resulting in further phosphorylation of the CD20 inner cytoplasmic chain and also phospholipase C-gamma. At the same time there is an upregulation of C-myc and myb messenger ribonucleic acid, an increase in adhesion molecule expression, and an upregulation of MHC class II proteins. The ultimate result is caspase 3 activation, causing cell apoptosis.

Results of studies with rituximab alone as first-line treatment of low-grade non-Hodgkin's lymphoma have been encouraging [92, 93], as well as inhibition of p38 kinase [94]. Rituximab has been also combined with conventional CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) for patients with intermediate grade or diffuse large-cell non-Hodgkin's lymphoma [95, 96].

Alemtuzumab is a mAb targeted at the CD52 antigen, found on the surface of most chronic lymphocytic leukemia (CLL) cells. It is particularly efficient in chemotherapy-resistant B-CLL. Binding of alemtuzumab to CD52 on target cells may cause cell death by 3 different mechanisms: complement activation, antibody-dependent cellular cytotoxicity, and apoptosis [97, 98].

In addition, mAbs can be used to deliver a toxin, such as the RFB4(dsFv)-PE38 (BL22), a recombinant immunotoxin containing an anti-CD22 variable domain (Fv) fused to truncated pseudomonas exotoxin [99]. CD22 antigen is found on the surface of hairy cell leukemia (HCL) cells. To target relapsed/refractory HCL, immunotherapy has been developed using anti-CD25 and anti-CD22 recombinant immunotoxins, or rituximab alone or combined with purine analogs. BL22 is now in phase I and II testing of relapsed/refractory HCL, achieving 47–61% complete remissions, several of them ongoing after 9-10 years [100].

Hematological malignancies show a wide variety of surface markers as potential target for mAbs targeting [91, 101–103]. In comparison to hematological malignancies, solid tumors have fewer specific targets for mAbs that are not cross-reactive with antigens on normal tissues.

In 2006 the FDA approved trastuzumab, the first monoclonal antibody for the treatment of a solid tumour, in HER2 overexpressing breast cancer [104]. Trastuzumab works in several ways: downregulation of HER2 receptor expression; inhibition of proliferation of human tumour cells that over-express HER2 protein; enhancing immune recruitment and antibody-dependent cell-mediated cytotoxicity (ADCC) against tumour cells that overexpress HER2 protein, and downregulation of angiogenesis factors. Trastuzumab also increases the effect of chemotherapy on breast cancer cells (on the average the response rate rises from 50% up to 85%), and it is currently used in combination with different chemotherapy regimens in metastatic disease, in adjuvant and neoadjuvant setting [105, 106].

It is important to point out that there are several evidences suggesting that blockade of signal transduction may not be the only mechanism of action of mAbs since a potential role of immunologic mechanisms in the therapeutic efficacy of ErbB-targeted mAbs (as opposed to TKI) has been reported. Among the variables known to play a role in the antitumor activity of TA-targeted mAbs, there is their ability to mediate lysis of tumor cells *in vitro* by NK cells, monocytes, and granulocytes in an ADCC way. The extent of lysis is in turn influenced by several variables, and they, or at least some of them, may contribute to the differential clinical response of patients treated with mAbs-based immunotherapy [107].

mAbs also bind complement, leading to direct cell toxicity, known as complement-dependent cytotoxicity (CDC).

Cetuximab is mAb effective for treatment of advanced colon cancer in combination with 5-fluorouracil, oxaliplatin, or irinotecan chemotherapy. It is also useful in locally advanced head and neck squamous-cell carcinoma when combined with radiotherapy or in recurrent head and neck cancer, combined with platinum-based chemotherapy [108].

Several papers have reported that the activity of cetuximab, as well as of panitumumab, is related to their link to the epidermal growth factor (EGFR) which prevents cancer cells from growing. In particular, it has been shown that these mAbs are effective only in patients whose cancer has no mutation of K-RAS gene, the so-called wild-type sequence. The mutation can be detected in about 40% of patients. The K-RAS mutations keep the EGFR always active so that its pathway can no longer be stopped by simply blocking the receptor [109].

Other mAbs have the function to enhance T-cell activation by blocking CTLA-4, a major negative regulator of T-cell-mediated responses. As we discussed previously, CTLA-4 is a homolog of CD28 that functions as an inhibitory receptor for B7 costimulatory molecules expressed on mature APCs. Anti-CTLA-4 mAbs with a much greater affinity for CTLA-4 than B7 may provide a survival advantage compared to vaccines or chemotherapy alone [90]. On the basis of these preclinical data, clinical trials have been initiated with two fully human anti-CTLA-4 mAbs, with different pharmacokinetic and pharmacodynamic profiles.

Anti-CTLA4 blocking antibodies [110] are effective in the treatment of malignant melanoma and may increase Th17 cells in peripheral blood of patients with metastatic melanoma. However, anti-CTLA-4 antibody therapy is associated with autoimmune toxicity, due to the augmented cellular proinflammatory activity, as consequence of the increase of Th17 cells and of the inhibition of Tregs function [17].

Tremelimumab is a human IgG2 anti-CTLA-4 mAb with a serum half-life of approximately 22 days, the same reported for endogenous human IgG2, which is currently under evaluation at escalating doses (from 3 to 15 mg/kg every three months) in several phase I studies in patients with metastatic tumors such as pancreatic, breast [111] and renal cell carcinoma in combination with conventional therapies. As a single agent, tremelimumab did not demonstrate a clinically significant activity in a phase II study of patients with refractory metastatic CRC [112]; it generated durable tumor responses in a phase I/II trial of patients with treated metastatic melanoma [113], but it failed to produce a survival advantage in a randomized phase III study compared to conventional chemotherapy with dacarbazine or temozolomide [20].

Ipilimumab is a fully human monoclonal antibody IgG1 with a shorter half-life, which was tested at a dose of 3 mg/kg with or without dacarbazine and at 10 mg/kg as monotherapy every 3 weeks in several phase II studies in metastatic melanoma patients showing a significant activity with durable remissions [114]. These results have been confirmed recently in the first randomized phase 3 trial [115] in patients with previously treated advanced melanoma with ipilimumab significantly prolonging median overall survival both as a single agent (10.1 months; $P < 0.003$) and combined with a gp100 vaccine (10.0 months; $P < 0.001$) compared with vaccine control (6.4 months). Even more noteworthy was the improvement in long-term survival at 24 months from 13.7% (gp100 alone) to 21.6% and 23.5% for the combination and single ipilimumab, respectively [115]. In addition some patients who progressed after an initial response (consisting of stable disease for more than six months, partial or complete response) were rechallenged within 28 days of documented progression with ipilimumab, showing a 50% response rate [114]. This pattern of delayed response is peculiar of anti-CTLA4 antibodies and is the reason why novel immune-related response criteria were developed, according to which progressive disease is defined as an increase $\geq 25\%$ in the sum of tumor diameters confirmed by two scans at least 4 weeks apart. However, anti-CTLA4 agents also exhibit a severe profile of adverse events including severe rash, grade 3-4 enterocolitis, hypophysitis, hepatitis, and more rarely, uveitis, pancreatitis, neuropathy, severe leucopenia, and red cell aplasia which are generally manageable and reversible if recognized early and treated promptly with corticosteroids [114]. Also ipilimumab produced encouraging results in phase I trails for patients with hormone-refractory prostate cancer, ovarian cancer, and non-Hodgkin's lymphoma and in phase II study of patients with metastatic clear cell renal carcinoma [114, 116]. It also significantly increased progression-free survival after conventional chemotherapy with carboplatin and paclitaxel in patients with untreated lung cancer [117]. On the basis of these data, anti-CTLA4 monoclonal antibodies represent one promising strategy to support and enhance the patient's natural antitumor response.

3.4. Antiangiogenic mAbs. Antiangiogenic drugs are biological therapies that stop tumors from creating their own blood vessels. There are different types of drugs that block blood vessel growth, including drugs that prevent growth factors from reaching cancer cells, drugs that block the growth factor inside the cell, and drugs that affect signals between cells. Vascular endothelial growth factor (VEGF) is one of the main proteins involved in angiogenesis [118, 119].

Bevacizumab, by blocking VEGF, can stop the receptors from sending signals necessary for blood-vessel growing. Once a receptor on a cell surface has been triggered and the pathway inside the cell activated, only tyrosine kinase inhibitors (TKIs), such as Sunitinib, can block signals that trigger the growth of new blood vessels.

Thalidomide is another antiangiogenic drug; even if its mechanism of action is still not well known, it seems to interfere with growth signals among cells. It is helpful for refractory multiple myeloma.

3.5. Conjugated mAbs. As discussed above, mAbs may carry other drugs or radiation directly to cancer cells. Monoclonal antibodies can be conjugated with anticancer drugs, radioisotopes, other biologic response modifiers, or other toxins. When the antibodies bind with antigen-bearing cells,

Table 1: Summary of some of the most promising drugs currently under investigation, with their target molecule and more promising diseases of application.

Class of products	Drug name	Target	Malignancies showing promising results
Monoclonal antibodies (mAbs)	*Ipilimumab* *Tremelimumab*	CTLA-4	Melanoma*, Non-Hodgkin's lymphoma, prostate cancer, renal cell cancer
	Rituximab	CD20	B-cell lymphoproliferative malignances
	Alemtuzumab	CD52	B-CLL
	Trastuzumab	HER2/neu	Breast cancer
	Cetuximab *Panitumumab*	EGFR	CRC, head and neck cancer, and others
	Bevacizumab	VEGF	CRC, metastatic breast cancer, NSCLC, advanced/metastatic renal cell carcinoma
Conjugated mAbs	*Tositumomab* *Ibritumomab*	CD20	B-cell lymphoproliferative malignances
Oncogene inhibitors	*Plexxikon*	BRAF	Melanoma
Vaccines	*Sipuleucel-T*	APC presenting prostatic antigens	Prostate cancer
	TroVax	APC presenting 5T4 epitope	Advanced CRC, renal cell carcinoma, prostate cancer
HSP90 inhibitors	*17-AAG* *geldanamycin*	HSP90	Various cancer

Abbreviations used in the table:
APC: antigen presenting Cell,
B-CLL: B-cell chronic lymphocytic leukemia,
CRC: coloRectal carcinoma,
EGFR: epidermal growth factor Receptor,
HCC: hepatocellular carcinoma,
HSP90: heat shock protein 90,
NSCLC: *non-small-cell lung carcinoma,*
VEGF: vascular epidermal growth factor,
*This agent as most of the others may also be used in combination with TAA-based vaccines, cytokines, and chemotherapy.

they deliver their load of drug directly to the tumour. Tositumomab and Ibritumomab are two new promising monoclonal antibodies, conjugated with radioisotopes, targeting CD20, that are still under investigation.

Antibody directed enzyme prodrug therapy (ADEPT) is a selective way for carrying an anticancer drug directly to cancer cells. The treatment is given in 2 steps. First, a mAb provided with an enzyme attached to it is administered; then, an inactive anticancer drug called a prodrug is given. When the prodrug and the enzymes meet in the cancer cell, the pro drug becomes active. This approach is still under investigation [120].

4. Conclusions

While the drugs reported above have clearly shown antitumor activity (a summary of some of the most promising is reported in Table 1), it is still possible to use these drugs in combination with TAA-based vaccines, cytokines, and chemotherapy.

While there are numerous immunotherapies with potential for destruction of human cancers, we are tempted to speculate that future goal of the field may be in a combination of techniques.

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Review Article

The Roles of Tumor-Derived Exosomes in Cancer Pathogenesis

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Exosomes are endosome-derived, 30–100 nm small membrane vesicles released by most cell types including tumor cells. They are enriched in a selective repertoire of proteins and nucleic acids from parental cells and are thought to be actively involved in conferring intercellular signals. Tumor-derived exosomes have been viewed as a source of tumor antigens that can be used to induce antitumor immune responses. However, tumor-derived exosomes also have been found to possess immunosuppressive properties and are able to facilitate tumor growth, metastasis, and the development of drug resistance. These different effects of tumor-derived exosomes contribute to the pathogenesis of cancer. This review will discuss the roles of tumor-derived exosomes in cancer pathogenesis, therapy, and diagnostic.

1. Introduction

Membranous vesicle shedding from live cells was first observed in the early 1980s and was proposed to be a mechanism through which cells discard inert debris [1–4]. Different types of membrane vesicles are secreted by cells, formed either at the surface of a blebbing plasma membrane or inside internal cellular compartments [5]. Among them, a population of nanosized membrane vesicles, termed "exosomes," has gained interest for their pleiotropic biological activity. Exosomes are defined as vesicles formed by "inward/reverse budding" of the limiting membrane of the multivesicular bodies (MVBs) in the late endocytic compartment and released upon the fusion of MVB with the plasma membrane [6, 7]. They are characterized by a size of 30–100 nm in diameter and a density of 1.13–1.19 g/mL in a sucrose gradient and can be sedimented at 100,000 ×g [5, 8]. Exosomes typically show a "cup-shaped" or "saucer-like" morphology when analyzed by electron microscopy. Exosome secretion is observed from most cell types under both physiological and pathological conditions, especially tumor cells and hematopoietic cells including reticulocytes [2, 4, 9, 10], dendritic cells (DCs) [11], B and T lymphocytes [12–15], platelets [16], mast cells [17, 18], and macrophages [19]. In addition, exosomes are also released by epithelial cells [20], fibroblasts [21], astrocytes, and neurons [22]. The extent of exosome secretion can be modulated in different cell types by either ligand cognition or stress conditions. For example, radiation treatment is able to increase the level of exosome secretion by tumor cells, a process possibly involving the activation of p53 and the subsequent upregulation of the transmembrane protein tumor suppressor-activated pathway 6 (TsAP6) [21, 23].

Exosomes contain cytosolic and membrane proteins derived from the parental cells. The protein content largely depends on their cellular origin and are generally enriched for certain molecules, including targeting/adhesion molecules (e.g., tetraspanins, lactadherin and intergrins), membrane trafficking molecules (e.g., annexins and Rab proteins), cytoskeleton molecules (e.g., actin and tubulin), proteins involved in MVB formation (e.g., Alix, Tsg101 and clathrin), chaperones (e.g., Hsp70 and Hsp90), signal transduction proteins (e.g., protein kinases, 14-3-3, and heterotrimeric G proteins) and cytoplasmic enzymes (e.g., GAPDH, peroxidases, and pyruvate kinases) [5, 8, 24]. Antigen presenting cell- (APC-) derived exosomes are also enriched in antigen-presenting molecules including MHC class I and class II complexes and costimulatory molecules [25]. Tumor-derived exosomes usually contain tumor antigens as well as certain immunosuppressive proteins such as FasL, TRAIL, or TGF-β [26]. In addition to proteins, functional RNA molecules including

mRNA and microRNAs have also been identified in exosomes [27–29].

Exosomes are now recognized as important mediators of cell-to-cell communication [30]. However, how these vesicles interact with and regulate the function of target cells remains largely unknown. Several types of interactions are proposed based on indirect evidence and *in vitro* studies, including (1) binding of vesicles to the surface of a recipient cell through exosomal adhesion molecules, or phosphatidylserine (PS)/lysophosphatidylcholine and cellular receptors (e.g., LFA1, TIM1 and TIM4); (2) direct fusion of vesicles with recipient plasma membrane after adhesion; or (3) internalization of vesicles into endocytic compartments through receptor-mediated endocytosis or phagocytosis [5]. Also, the symmetrical phatidylethanolamine repartitions in exosome membranes may facilitate their absorption, but not fusion with target cells such as DCs [31].

The interaction between exosomes and target cells can lead to direct stimulation of target cells via surface-expressed growth factors or bioactive lipids, transfer of membrane receptors, or delivery of proteins to target cells. Also, the presence of mRNA and microRNA, termed "exosomal shuttle RNA," in exosomes suggests that genetic material exchange could be an additional level of exosome-mediated communication between cells [27].

There is still some confusion in describing different types of vesicles secreted by cells. The terms "exosomes," "microvesicles," and "membrane particles" are sometimes used interchangeably. Generally, the term "microvesicles" refers to vesicles shed from the plasma membrane, have a relatively larger size (100–1000 nm) than exosomes and can be sedimented at 10,000 ×g. The term "membrane particles" refers to vesicles that also originate from plasma membrane, but have a small size similar to exosomes [5]. In this review, we will focus specifically on the various effects of exosomes on tumorigenesis.

2. Antitumorigenic Role of Tumor-Derived Exosomes

2.1. Immunogenic Properties and Tumor Exosome-Based Cancer Vaccines. The protein composition of exosomes largely reflects that of their parental cells and thus shows cell-type specificity. In particular, tumor-derived exosomes contain tumor-specific antigens expressed in the parental tumor cells. Enrichment of tumor antigens such as melan-A [32], Silv [33], carcinoembryonic antigen (CEA) [34], and mesothelin [35] is observed in tumor-derived exosomes when compared with whole cell lysates [26]. The observation that most tumor cells release exosomes containing tumor antigens suggests that tumor exosome-based cancer vaccines could be developed. Indeed, tumor-derived exosomes have been used as a source of tumor antigens to pulse DCs, resulting in the transfer of tumor antigens to DCs that were able to induce CD8+ T cell-dependent antitumor effects in mice [33]. In a similar human *ex vivo* model system, DCs pulsed with exosomes derived from malignant effusions expressing tumor antigens cross-present the antigens to antigen-specific cytotoxic T lymphocytes (CTLs) [32]. Recently, it was reported that tumor exosome-loaded DCs effectively elicited tumor-specific CD8+ CTL response against autologous tumor cells in patients with malignant gliomas [36].

Direct application of tumor-derived exosomes for the enhancement of antitumor immunity also has been investigated. It was reported that tumor-derived exosomes could induce specific antitumor responses when the parental tumor cells were genetically modified to express pro-inflammatory cytokines such as IL-18, IL-12, and IL-2 [37–39] or when the parental tumor cells were subjected to stress conditions. For example, heat-shocked lymphoma cells release exosomes with increased levels of MHC and co-stimulatory molecules and induce efficient antitumor T cell immunity [40]. Additionally, exosomes derived from heat-shocked tumor cells were observed to contain elevated levels of Hsp70 and elicit Th1-polarized immune responses *in vitro* and *in vivo* in both autologous and allogeneic murine models, suggesting that these exosomes can stimulate antitumor immunity in an MHC-independent manner [41]. Moreover, it was reported that exosomes derived from tumor cells engineered to express membrane-bound Hsp70 stimulate Th1 and CTL antitumor immunity more efficiently than those derived from heat-shocked tumor cells [42]. Heat-stressed tumor cells were also found to release exosomes with enriched chemokines that could attract and activate DCs and T cells more potently and induce specific antitumor immune response more efficiently than exosomes from untreated tumor cells [43]. Furthermore, surface targeting of antigens to exosome membranes can enhance the immunogenicity of tumor-derived exosomes, as membrane targeting of the superantigen staphylococcal enterotoxin A (SEA) [44] or chicken egg ovalbumin (OVA) [45] resulted in enhanced CTL activity and delayed tumor growth.

The promising results obtained in animal tumor models led to several phase I clinical trials using tumor-derived exosomes or exosome-pulsed DCs as cancer vaccines [46, 47]. However, it is important to note that in animal experiments, effective antitumor immune responses were mostly achieved when tumor-derived exosomes were loaded onto matured APCs or were modified to contain high levels of pro-inflammatory factors or stress proteins. The representative studies on the immunogenicity of tumor-derived exosomes and tumor exosome-based cancer vaccines are listed in Table 1.

2.2. Induction of Tumor Cell Apoptosis. In addition to the potential immunostimularoty effects, a proapoptotic function of tumor-derived exosomes directly on tumor cells was also reported. Exosome-like vesicles produced by human pancreatic tumor cells were reported to increase Bax and decrease Bcl-2 expression, inducing tumor cells toward mitochondria apoptotic pathway. These exosomes also induced phosphatase and tensin homolog (PTEN) and glycogen synthase kinase-3β (GSK-3β) activation and decreased pyruvate dehydrogenase activity in treated cells, sequestered β-catenin-dependent survival pathway, and counteracted the constitutively activated phosphatidylinositol 3-kinase/Akt survival pathway to drive tumor cells toward apoptosis [48].

Table 1: Representative studies on the immunogenicity of tumor-derived exosomes and tumor exosome-based cancer vaccines.

Parental tumor type/exosome source	Exosome application/modification	Model	Results	References
Mouse mammary adenocarcinoma, melanoma, mesothelioma, mastocytoma, human melanoma	BMDC pulsed with exo were injected into mice with established tumor	Mouse	Exo transfer tumor antigen to DC, induce CD8+ T cell-dependent antitumor effects on both syngeneic and allogeneic mouse tumors	[33]
Malignant effusions of melanoma patients	MDDCs-pulsed exo were used to stimulate lymphocytes	Human *ex vivo*	DCs pulsed with exo cross-present mart-1 antigen to and expand antigen-specific CTLs	[32]
Human malignant glioma	Human DCs were incubated with exo	Human *ex vivo*	DCs incubated with exo activate glioma-specific CTL which kills autologous glioma cells *in vitro*	[36]
Human CEA+ colon, lung carcinoma	Exo were isolated from heat-stressed tumor cells	Mouse, Human *ex vivo*	Exo immunization efficiently prime antigen-specific CTL with antitumor effects in mice; exo-pulsed autologous DCs from CEA+ cancer patients induce antigen-specific CTL response	[34]
Mouse B lymphoma	Parental cells were heat-shocked	Mouse	Exo induce DC maturation and stimulate both protective and therapeutic antitumor immune responses	[40]
Mouse colon carcinoma and melanoma	Parental cells were heat-treated	Mouse	Exo contain elevated levels of Hsp70, elicit Th1 response and therapeutically regress established autologous and allogeneic tumors	[41]
Mouse melanoma	Parental cells were engineered to express membrane-bound Hsp 70	Mouse	Exo stimulate Th1 and CTL response more efficiently than exo derived from heat-shocked cells expressing cytoplasmic Hsp70	[42]
Mouse lung carcinoma	Parental cells were heat-stressed	Mouse	Exo contain enriched chemokines, attract/activate DCs and T cells more potently and induce antitumor response	[43]
Human CEA+ tumor cells	Parental cells were transfected with AdhIL-18	Human *ex vivo*	Exo/IL-18 chemoattract DCs and T cells and enhance Th1 cytokine release. Exo/IL18-pulsed DCs induced potent CTL response	[37]
Mouse OVA+ thymoma	Parental cells were transfected with AdmIL-12	Mouse	Vaccination of exo/IL-2 induces antigen-specific Th1 and CTL responses and inhibits tumor growth	[39]
Human renal cancer	Parental cells were modified to express GPI-IL-12	*In vitro*	Exo/IL-12 promote IFN-γ release and the induction of antigen-specific CTLs	[38]
Mouse lymphoma	Exo were surfaced anchored with the superantigen SEA by protein transfer	Mouse	Immunization with exo/SEA-TM efficiently inhibits tumor growth and induces tumor-specific CTLs	[44]
Mouse fibrosarcoma	OVA antigen was targeted to exo membrane by transfecting parental cells with OVA coupled to lactadherin C1C2 domain	Mouse	Tumors secreting exo-bound OVA elicit a stronger anti-OVA response and grow slowly *in vivo*	[45]
Human ovarian cancer ascites	Exo were purified from malignant ascites and quality assessed	Preceding of a clinical trial	A method for the preparation of GMP-grade exosomes used in combination of mature DCs for a clinical trial is described	[46]
Ascites from colorectal cancer patients	Exo were purified and used to immunize patients either alone or with GM-CSF	Phase I clinical trial	Exo therapy is well-tolerated; exo plus GM-CSF induce beneficial tumor-specific CTL responses in patients with colorectal cancer	[47]

Abbreviations: Exo, exosomes; MDDCs: monocyte-derived DCs; Ad: adenovirus; GM-CSF: granulocyte-macrophage colony-stimulating factor.

The interaction of these exosomes with pancreatic cancer cells also led to decreased expression of the intranuclear target of the Notch-1 signaling pathway, thereby inhibiting the Notch-1 survival pathway and activating the apoptotic pathway [49].

Despite the potential antitumor effects of tumor-derived exosomes, it is still unclear whether the constant production of exosomes by tumor cells is beneficial or harmful for their own survival *in vivo*. Notably, in cancer patients with advanced disease, tumor-derived exosomes are produced abundantly in the tumor microenvironment, however effective immunostimulatory or antitumor effects of these vesicles are rarely observed. In fact, there is substantial evidence supporting a role of tumor-derived exosomes in preventing antitumor immune responses and promoting tumorigenesis.

3. Protumorigenic Role of Tumor-Derived Exosomes

3.1. Immunosuppressive Properties. The observation that membrane vesicles shed from murine melanoma cell lines inhibited the expression of the immune response region-associated antigen by macrophages provided early evidence that tumor-derived membrane vesicles is a possible mechanism whereby tumor-bearing hosts become immunocompromised [50]. More recently, diverse immunosuppressive effects of tumor-derived exosomes have been identified. Tumor-derived exosomes were shown to directly suppress the activity of effector T cells. Certain tumor cell lines can produce exosomes expressing death ligand such as FasL and TRAIL, both of which can trigger the apoptotic death of activated T cells [51, 52]. Additionally, Epstein-Barr Virus- (EBV-) infected nasopharyngeal carcinoma (NPC) was shown to release exosomes containing high amounts of galectin-9, which induces apoptosis of mature Th1 lymphocytes when interacting with the membrane receptor Tim-3. These exosomes prevent galectin-9 from being proteolytically cleaved and thus induce massive apoptosis of EBV-specific CD4+ cells [53]. Moreover, ovarian tumor-derived exosomes were found to down-modulate CD3-ζ chain expression and impair TCR signaling [54], suggesting that tumor-derived exosomes can also downregulate T cell function in addition to direct killing. In addition, NKG2D-dependent cytotoxicity of NK cells and CD8+ T cells was inhibited by NKG2D ligand-containing exosomes derived from human breast cancer and mesothelioma cell lines [55, 56]. Similarly, murine mammary carcinoma exosomes were shown to promote tumor growth *in vivo* by suppressing NK cell function [57]. Taken together, these observations suggest that tumor-derived exosomes can negatively regulate the effector arm of the immune system, in particular T cells and NK cells.

Tumor-derived exosomes can also target myeloid cells to modulate their differentiation and function. Exosomes derived from human melanoma cell lines and colorectal carcinoma cell lines were shown to skew monocyte differentiation into DCs toward the generation of myeloid-derived suppressor cells (MDSCs) and exert TGF-β1 mediated suppressive activity on T cells *in vitro*. Interestingly, significant expansion of MDSC-like CD14+HLA-DR-/low and TGF-β-secreting cells was also found in the peripheral blood of late-stage melanoma patients and high levels of MDSCs is usually associated with poor responses to tumor vaccines [58]. Similar effects were observed in mouse models where exosomes produced by murine mammary carcinoma cells and melanoma cells targeted CD11b+ myeloid precursors in the bone marrow (BM) and inhibited the differentiation of BMDCs by inducing IL-6 in these precursor cells [59]. These myeloid cells were found to switch their differentiation pathway toward an MDSC phenotype and promote tumor growth, dependent on the prostaglandin E2 and TGF-β molecules present on tumor-derived exosomes [60]. MyD88 also appears to play a pivotal role in melanoma exosome-mediated MDSC expansion and tumor metastasis [61]. Similarly, the membrane-associated Hsp72 on tumor-derived exosomes was reported to mediate STAT3-dependent immunosuppressive function of MDSCs by triggering STAT3 activation in a Toll-like receptor- (TLR-) 2/MyD88-dependent manner [62], although the role of TLR2 in this process remains controversial [63, 64].

The effect of tumor-derived exosome on BM cells is thought to be a coevolutionary strategy of the primary tumor and the tumor microenvironment [65]. Alteration of BM cell behavior by tumor-derived exosomes can be mediated by proteins or by transfer of genetic materials, such as mRNA and microRNA, between tumor cells and BM cells, thereby influencing the function of future populations of BM cells. RNA transfer to BM cells by microvesicles released from other tissue/cell sources and the transcription of tissue-specific mRNA in BM cells has been observed [66, 67], suggesting that a similar effect also can be mediated by tumor-derived exosomes.

In addition, tumor-derived exosomes can also support the function of regulatory T (Treg) cells. For example, human tumor-derived exosomes were found to selectively impair the IL-2 response to cytotoxic effector cells while supporting Treg cell activities through a TGF-β-dependent mechanism [35]. Tumor-derived exosomes were also reported to induce, expand, and upregulate the suppressor functions of human Treg cells as well as enhance their resistance to apoptosis via a TGF-β- and IL-10-dependent mechanism [68]. A similar effect was observed with exosomes derived from the malignant effusion of cancer patients as these exosomes, most of which have a tumor origin, helped maintain the number and suppressive function of Treg cells [69].

Given that tumor-derived exosomes are capable of altering APC function and enhancing regulatory cell activity while at the same time are a source of tumor antigen, it is tempting to speculate that tumor-derived exosomes may also have the ability to promote tolerance to tumor-specific antigens. Indeed, we have demonstrated that tumor-derived exosomes bearing a model tumor antigen were able to induce antigen-specific immunosuppression in a murine delayed-type hypersensitivity model. We proposed a mechanism that tumor-derived exosomes provide tumor antigens to DCs as well as condition DCs toward a suppressive/tolerogenic phenotype, resulting in the downregulation of antigen-specific immune responses [70].

3.2. Facilitation of Tumor Invasion and Metastasis. In addition to attenuating different branches of the antitumor immunity to help tumor cells survive immunosurvelliance, tumor-derived exosomes have also been implicated in facilitating tumor invasion and metastasis. By stimulating angiogenesis, modulating stromal cells, and remodeling extracellular matrix, tumor-derived exosomes have been found to contribute to the establishment of a premetastatic niche, generating a suitable microenvironment in distant metastatic sites [65].

Early proteomic analysis of mesothelioma cell-derived exosomes detected the presence of strong angiogenic factors that can increase vascular development in the neighborhood of tumor [71]. Melanoma-derived exosomes were also found to stimulate endothelial signaling important for tissue matrices remodeling and endothelial angiogenesis [72]. Moreover, it was recently reported that melanoma exosomes injected locally preferentially homed to sentinel lymph nodes and prepared the lymph nodes to become remote niches conducive to the migration and growth of melanoma cells through the induction of molecular signals for melanoma cell recruitment, extracellular matrix deposition, and vascular proliferation [73]. Consistent with these observations, it was reported that mice pretreated with melanoma exosomes have a significant acceleration of melanoma metastasis in the lung [61].

Tetraspanins, which are constitutively enriched in exosomes, have been found to contribute to exosome-mediated angiogenesis. It was reported that exosomes derived from a pancreatic tumor line overexpressing D6.1A, a tetraspanin associated with poor prognosis in patients with gastriointestinal cancer, strongly induced endothelial cell branching *in vitro* and angiogenesis *in vivo* in a rat model [74]. Tumor-derived D6.1A stimulates the secretion of matrix metalloproteinase and urokinase-type plasminogen activator, enhances the expression of vascular endothelial growth factor expression in fibroblasts, and upregulates the expression of endothelial growth factor receptor as well as D6.1A in sprouting endothelium. Moreover, the D6.1A-expressing cell promoted angiogenesis independent of cell-cell contact, highlighting the potential role of D6.1A-enriched tumor-derived exosomes in inducing systemic angiogenesis. Recently, exosomal Tspan8 (D6.1A) was found to contribute to the selective recruitment of proteins and mRNA into exosomes, including CD106 and CD49d, both of which were implicated in the binding and internalization of exosomes by endothelial cells. Induction of several angiogenesis-related genes, together with enhanced endothelial cell proliferation, migration sprouting and maturation of endothelial cell progenitors, were seen upon exosome internalization [75]. Tumor-derived exosomes were also found to incorporate the Notch ligand Delta-like 4 (Dll4) and transfer the Dll4 protein into the cell membrane of host endothelial cells, resulting in the inhibition of Notch signaling and the switch of endothelial cell phenotype toward tip cells. This further results in an increase in vessel density *in vitro* and an increase in branching *in vivo* [76].

Another pronounced effect of tumor-derived exosomes is their ability to modulate the function of stromal cells such as fibroblasts. It was recently shown that exosomes produced by a certain type of cancer cells contain TGF-β on their surface in association with betaglycan and can trigger SMAD-dependent signaling. Exosomal delivery of TGF-β is capable of driving the differentiation of fibroblasts into myofibroblasts, whose enrichment in solid tumor represents an altered stroma that usually supports tumor growth, vascularization, and metastasis. Exosomal TGF-β delivery is also qualitatively different from soluble TGF-β in that they induce a more significant elevation of fibroblast FGF2 production [77]. These observations suggest another protumorigenic role of tumor-exosomal TGF-β in addition to their immunosuppressive functions. However, it was also noted that TGF-β is not universally present on exosomes derived from all cancer cells.

Furthermore, exosomes shed by gynecologic neoplasias, including ovarian cancer and breast cancer cells, were found to contain metalloproteinases that have proteolytic activity. These exosomes can increase extracellular matrix degradation and augment tumor invasion into the stroma [78–80]. It was suggested that CD44 is required for the assembly of a soluble matrix which may serve as an exosome carrier and/or a reservoir for growth factors, chemokines, and proteases needed for tumor cell embedding and growth. Selective knockdown of CD44 resulted in a striking reduction of the metastasizing capacity of the highly metastatic tumor in a rat pancreatic adenocarcinoma model [81].

Interestingly, tumor-derived microvesicles, which are mostly shed from tumor plasma membrane, were found to have certain effects similar to exosomes, such as stimulating angiogenesis [82, 83], modifying stromal cells [84], and degrading extracellular matrix [85–87], possibly because that they have comparable compositions and that the proteins involved are present on both types of vesicles. However, the vesicles reported to have a procoagulant effect that correlates with an increased risk of cancer-associated thromboembolism have been mostly microvesicles, rather than exosomes, likely because the tissue factors and other contents with procoagulant activity such as PS and Mucin 1 mostly reside in the cell surface membrane. Those microvesicles are also thought to play an important role in supporting tumor growth by inducing the local fibrin deposits associated with many solid tumors [88–92].

3.3. Transport of RNAs and Proteins for Tumor Survival and Growth. The intercellular exchange of proteins and genetic materials via exosomes is a potentially effective approach for cell-to-cell communication within the tumor microenvironment [93]. In particular, transport of mRNAs and microRNAs, from tumor cells to neighboring cells could have significant effects on tumorigenesis. Glioblastoma-derived exosomes were reported to transport mRNA into recipient cells where it is functionally translated. These exosomes stimulated glioma cell proliferation and promoted tumor growth [28]. The let-7 microRNA family was found to be selectively released in exosomes in a metastatic gastric cancer cell line. Since the *let-7* genes target oncogenes including RAS and HMGA2 and generally play a tumor-suppressor role, the release of let-7 microRNA via exosomes

could deliver oncogenic signals and promote metastasis [94]. Moreover, exosomes can also be utilized by human tumor virus for disseminating viral materials. For example, exosomes released from NPC cells with latent EBV infection contain EBV latent membrane protein 1 (LMP1) and viral microRNAs. These exosomes were able to transfer LMP1 into recipient cells and activate growth-signaling pathway [95]. Similarly, it was reported that the viral BART miRNAs are released from EBV-infected NPC cells into exosomes. These viral microRNAs could be detected in blood plasma samples from NPC xenografted nude mice as well as NPC patients, suggesting that exosomes enable these viral miRNAs to diffuse from the tumor site to the peripheral blood [96].

Tumor-derived exosomes may also transport apoptosis-inhibitory proteins induced under stress conditions to promote tumor survival. For example, survivin, a member of the inhibitor of apoptosis protein family, can be absorbed by cancer cells from extracellular media and inhibit their apoptosis following genotoxic stress as well as increase their replicative and metastatic ability [97]. It was found that survivin was released into exosomes from cervical carcinoma cells at a significantly higher level after irradiation, suggesting a potential exosome-mediated self-protective mechanism of these cancer cells [98].

3.4. Drug Interference. The protumorigenic role of tumor-derived exosomes is also reflected by their active participation in drug resistance through several mechanisms. One mechanism is by drug exportation via the exosome pathway. In human ovarian carcinoma cells that stably acquired resistance to the cancer chemotherapy drug cisplatin, the lysosome compartment, where the drug usually accumulates, was reduced with more exosomes released compared to cisplatin-sensitive cells. Moreover, when the cells were loaded with cisplatin, exosomes released from cisplatin-resistant cells contained 2.6-fold more platinum than those released from cisplatin-sensitive cells, suggesting that exosome secretion can be utilized by cancer cell to export anticancer drugs [99]. A similar effect was also observed in melanosomes, a type of lysosome-related organelles in pigmented cells such as melanoma cells [100]. One of the mechanisms by which lysosomal vesicles sequester cytotoxic drugs is increased acidification and treatment with proton pump inhibitors inhibited the acidification process and increased the sensitivity of tumor cells to chemotherapy drugs [101]. In addition, exosomes can also function to neutralize antibody-based drugs. Exosomes secreted by HER2-overexpressing breast carcinoma cell lines express a full-length HER2 molecule, enabling them to bind to the HER2 antibody Trastuzumab both *in vitro* and *in vivo*. The exosome-antibody interactions inhibit the overall effect of Trastuzumab on the proliferation of cancer cells by reducing antibody binding to cancer cells [102]. Such antibody sequestration was also demonstrated to reduce the antibody-dependent cytotoxicity effect on tumor cells by immune effector cells [103].

Taken together, tumor-derived exosomes exert protumorigenic effects via pleiotropic mechanisms (Figure 1). However, it is important to note that each of the numerous effects of exosomes reported was observed from exosomes derived from only a few of a wide variety of cancerous cell lines or types. Whether exosomes derived from a given tumor will have the sufficient complexity to confer multiple suppressive functions still needs to be determined [104]. It is likely that the predominant regulatory role of exosomes depends on their molecular phenotype and cell specificity. In addition, environmental factors could also play an important role in determining the behavior and immunological impact of tumor-derived exosomes.

4. Clinical Relevance of Tumor-Derived Exosomes

As discussed above, tumor-derived exosome-pulsed DCs, tumor-derived exosomes, and exosomes isolated from malignant ascites all have been investigated for their ability to elicit antitumor immune response in patients. However, although these clinical approaches appear to be safe, there has been a lack of clinical efficacy of exosome-based vaccines in contrast to the promising results obtained in many animal tumor models. Because of their potential immunosuppressive properties, direct administration of tumor-derived exosomes may actually result in promoted tumor growth. Therefore, clinical studies have focused on the use of tumor-derived exosome-loaded mature DCs [46] or ascites-derived exosomes [47], which may include both APC- and tumor-derived exosomes, together with proinflammatory factors. Still, the limited number of clinical trials and patients recruited prevents a conclusive evaluation of their efficacy and prospect.

The protumorigenic potential of tumor-derived exosomes in cancer patients is supported by the observations that in patients with breast or ovarian cancer, the level of circulating exosomes and exosomes with tumor markers is much higher than nonmalignant individuals and increases with tumor progression [29, 105], and that exosomes isolated from the sera of patients with oral or ovarian cancer can impair T lymphocytes function and induce their apoptosis [54, 106]. Therefore, it has been proposed that removing immunosuppressive tumor-derived exosomes from the blood circulation of a cancer patient would improve antitumor immune response and delay the progression and spread of malignancy. A novel hollow-fiber cartridge (Hemopurifier) system which is able to selectively deplete circulating virus using a lectin-based resin with high affinity for glycosylated viral surface proteins was developed by the San Diego biotechnology company Aethlon Medical [107]. Effective removal of HIV particles has been demonstrated [108–110] and this system has become an attractive device for depletion of exosomes, which have a size similar to viral particles and are also highly glycosylated on their membrane proteins. The selective removal of exosomes can be enhanced by attaching antibodies against exosome surface proteins onto the resin of the cartridge. However, there are still technical barriers in how to carefully distinguish tumor-derived from nontumor-derived exosomes and concerns such as the physiological outcome of removing all exosome-like vesicles in the blood.

On the other hand, tumor-derived exosomes containing tumor-specific protein and microRNA profiles have been

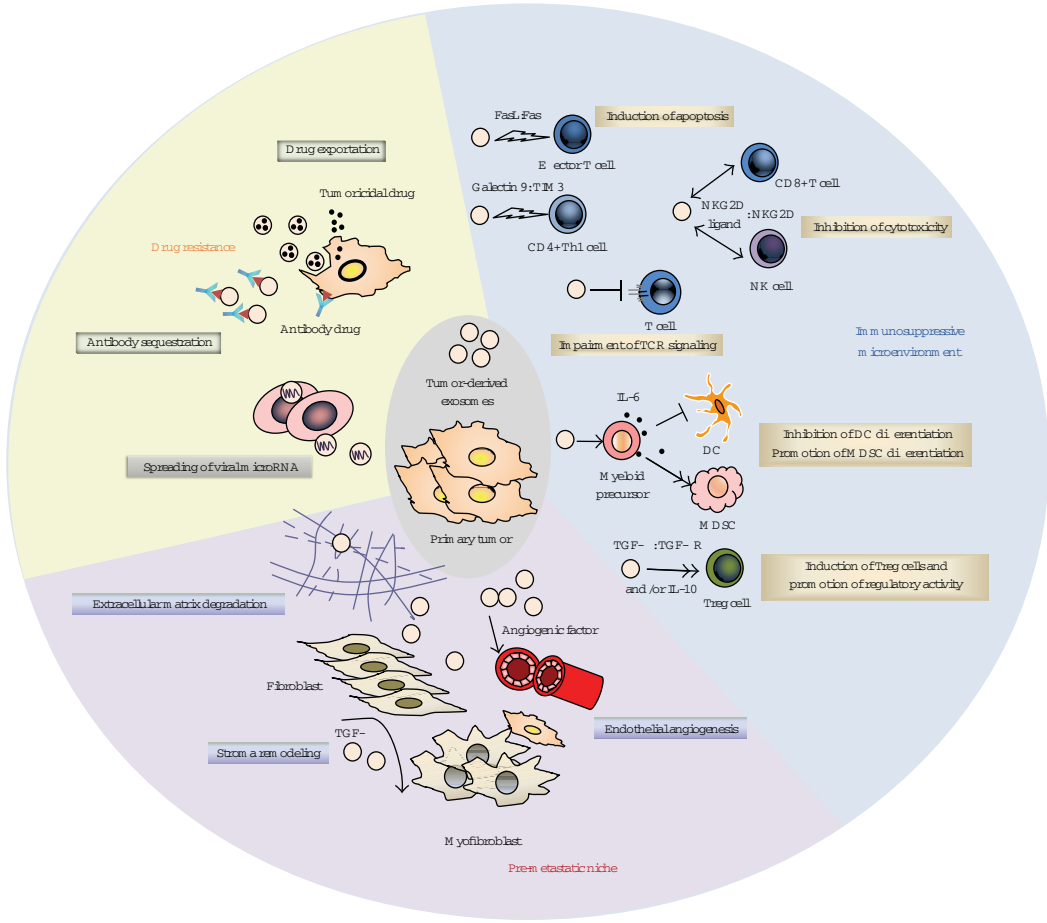

Figure 1: The protumorigenic role of tumor-derived exosomes. Tumor-derived exosomes help create an immunosuppressive tumor microenvironment by inducing apoptosis and impairing the function of effector T cells and NK cells, skewing DC differentiation into MDSCs as well as promoting Treg cell activity. They also contribute to the establishment of a pre-metastatic niche by enhancing angiogenesis, remodeling stromal cells, and promoting extracellular matrix degradation. Tumor-derived exosomes also function as delivery vehicles to transfer microRNA and mRNA to neighboring cells. Moreover, tumor-derived exosomes can help tumor cells develop drug resistance by exporting tumoricidal drugs or neutralizing antibody-based drugs.

proposed to be cancer diagnostic markers. Early detection of cancer could be easily performed using exosomes isolated from body fluids such as blood plasma, serum, and urine. Evidence supporting this approach include: (1) ovarian cancer-associated expression of claudin proteins can be detected in the circulating vesicles of a majority of ovarian cancer patients [111], (2) in breast cancer patients increasing levels of circulating vesicles expressing CEA and the cancer antigen 15-3 is correlated with increasing size of tumors [105], (3) exosomes expressing tumor markers can be isolated from the sera of ovarian cancer patients and the amount increases along with tumor progression [29]; and (4) in glioblastoma patients, mRNA variants and microRNAs characteristic of gliomas could be detected in serum vesicles [28]. However, it was also found that not in all cases tumor-derived exosomes were present in the blood circulation [112]. In a study on tumor-derived exosomes in the serum of glioblastoma patients, tumor-specific EGFRvIII was detected in serum exosomes in 7 out of 25 patients [28]. We recently demonstrated that tumor-derived exosomes with a chimeric membrane surface tag could not be detected in plasma-derived exosomes of mice bearing subcutaneous melanoma, possibly due to the rapid uptake of tumor-derived exosomes by APCs in the tumor microenvironment before they have access to the blood circulation (unpublished data). Therefore, different types of tumor and possibly different tumor growth patterns may both affect the accumulation of tumor-derived exosomes in peripheral circulation. Thus cautious interpretation is needed when using the presence of tumor-derived exosomes in body fluids as cancer diagnostic markers.

5. Conclusion

Increasing evidence suggests that tumor-derived exosomes can confer either antitumorigenic or protumorigenic effects. These seemingly controversial effects can be the results of complex interactions between exosomes, responding cells, and environmental factors. In cancer patients, the immunostimulatory or immuosuppressive effects of tumor-derived

exosomes may also depend on the stage of cancer progression as well as the immune status. Notably, as close replicas of their parental cells, tumor-derived exosomes are well positioned to transmit the detrimental effects of tumor cells onto the immune system to facilitate their survival, growth, and metastasis. Therefore, a better understanding of the roles of tumor-derived exosomes in cancer pathogenesis is needed to further improve anti-cancer therapeutics as well as exosome-based cancer diagnostics.

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Review Article

Antitumor Immunity Produced by the Liver Kupffer Cells, NK Cells, NKT Cells, and CD8$^+$ CD122$^+$ T Cells

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Mouse and human livers contain innate immune leukocytes, NK cells, NKT cells, and macrophage-lineage Kupffer cells. Various bacterial components, including Toll-like receptor (TLR) ligands and an NKT cell ligand (α-galactocylceramide), activate liver Kupffer cells, which produce IL-1, IL-6, IL-12, and TNF. IL-12 activates hepatic NK cells and NKT cells to produce IFN-γ, which further activates hepatic T cells, in turn activating phagocytosis and cytokine production by Kupffer cells in a positive feedback loop. These immunological events are essentially evoked to protect the host from bacterial and viral infections; however, these events also contribute to antitumor and antimetastatic immunity in the liver by activated liver NK cells and NKT cells. Bystander CD8$^+$CD122$^+$ T cells, and tumor-specific memory CD8$^+$T cells, are also induced in the liver by α-galactocylceramide. Furthermore, adoptive transfer experiments have revealed that activated liver lymphocytes may migrate to other organs to inhibit tumor growth, such as the lungs and kidneys. The immunological mechanism underlying the development of hepatocellular carcinoma in cirrhotic livers in hepatitis C patients and liver innate immunity as a double-edged sword (hepatocyte injury/regeneration, septic shock, autoimmune disease, etc.) are also discussed.

1. Introduction

The liver is the largest organ in vertebrates. Cumulative evidence has indicated that not only the fetal liver but also the adult liver is an important immune organ. The livers in adult mice contain c-kit$^+$ pluripotent hematopoietic stem cells, which are located in the perisinusoidal Disse spaces, and give rise to all lineages of leukocytes and red blood cells [1–3]. c-kit hematopoietic stem cells have also been identified in adult human livers [4]. When B cell- and T cell-deficient SCID mice were lethally irradiated and received bone marrow cells as well as liver mononuclear cells (MNCs) (but not splenocytes) from normal mice, the SCID mice could survive, and the thymus, liver leukocytes, splenocytes, and lymph nodes and bone marrow cells were all reconstituted [1]. The administration of purified c-kit$^+$ hematopoietic stem cells from either bone marrow or liver MNCs into SCID mice also reconstituted leukocytes in all organs [1]. In addition, liver Kupffer cells comprise 80% of the macrophage lineage cells in the whole body, and most bacteria that enter the blood stream accumulate in the liver and are killed by these Kupffer cells. In addition, other innate immune lymphocytes, NK cells [5, 6], and T cells with intermediate levels of TCR (TCR^{int} cells) are abundantly present in the liver [7], which are rarely seen in other organs and peripheral blood.

Among mouse TCR^{int} cells in the liver, 2/3 are CD122 (IL-2 receptor β)$^+$NK1.1$^+$ NKT cells and 1/3 are NK1.1$^-$CD122$^+$ T cells [3, 8–10]. The NK1.1$^+$ NKT cells are dependent on an MHC class-I like molecule, CD1d, for their development, express an invariant Vα14Jα18/Vβ8 gene product for their T cell receptor (TCR), and have a phenotype of CD4 or CD4$^-$CD8$^-$(double negative, DN) (afterwards, NKT cells) [10, 11]. On the other hand, NK1.1$^-$CD122$^+$ T cells are MHC class-I dependent for their development, and predominantly (11%) express the Vα11 gene product for their TCR [10] and have a phenotype of CD8 or DN (2/3 are CD8$^+$ and 1/3 are DN) (afterwards, CD8$^+$CD122$^+$ T cells). Since CD8$^+$CD122$^+$ T cells are also present in athymic nude mice and increase age-dependently in nude and normal mice, they may be of extrathymic origin.

Under physiological conditions, most MNCs (including Kupffer cells, NK cells, TCR^{int} cells) exist in the sinusoidal space in the liver parenchyma. Kupffer cells tightly adhere

Table 1: NKT cells are IL-12-induced antimetastatic effectors.

Mouse strain/treatment	Tumor	Site of metastasis tested	Number of tumor metastases		
			Control	IL-12 treated	% inhibition
BALB/c + IL-12	RL 1	liver	216 ± 24	28 ± 2	87%*
	Colon 26	lung	125 ± 25	16 ± 10	87%*
DBA/2 + lL-12	P815	liver	173 ± 12	10 ± 1	94%*
C57BL/6 + IL-12	B16	lung	61 ± 16	5 ± 1	91%*
	EL4	liver	106 ± 22	17 ± 7	84%*
C57BL/6 bg/bg + IL-12	EL4	liver	107 ± 17	16 ± 6	85%*
C57BL/6 + IL-12	EL4	liver	96 ± 18	15 ± 4	84%*
	3LL	lung	122 ± 26	25 ± 5	80%*
C57BL/6 + αAGM1 Ab + IL-12	EL4	liver	102 ± 24	22 ± 5	78%*
	3LL	lung	128 ± 32	33 ± 8	74%*
C57BL/6 + αNK1.1 Ab + IL-12	EL4	liver	152 ± 26	130 ± 20	14%
	3LL	lung	204 ± 36	156 ± 28	24%

The mice were inoculated i.v. with syngeneic tumors. Data of tumor metastasis and % of inhibition are shown as mean ± SD from six to ten mice in each group. $^*P < .01$. αAGM1 Ab: antiasialo GM1 antibody; αNK1.1 Ab: anti-NK1.1 antibody.

to sinusoidal endothelial cells, and NK/NKT cells are often in contact with these Kupffer cells and may normally elicit immunological functions to eliminate exogenous pathogens present in liver sinusoids that enter from portal vein and the systemic circulation. However, the localization of these MNCs is altered under pathological conditions. In human viral hepatitis or autoimmune hepatitis, a large number of lymphocytes infiltrate into the portal areas (where the portal vein, hepatic artery, and bile duct exist) and cause periportal inflammation. The experimental hepatitis model induced by α-galactosylceramide (α-GalCer, see Section 3) in mice leads to pathological findings similar to human viral hepatitis, such as piecemeal necrosis and apoptotic Councilman bodies in and around the portal areas, although MNCs also increased in sinusoids. These findings suggest that the antigen activation process may be initiated in and around portal areas.

NKT cells are mainly confined in the liver, and the proportion of NKT cells in liver MNCs remains constant regardless of the age of the mouse, whereas the $CD8^+CD122^+$ T cells constantly increase in the liver, as well as in the periphery, in an age-dependent manner [10]. In addition, both TCR^{int} cells display a potent IFN-γ producing capacity and antitumor cytotoxicity [12]. Notably, DN T cells with the intermediate TCR expanded in the liver, spleen, and lymph nodes in autoimmune MRL-*lpr/lpr (lpr)* mice may be an abnormal counterpart to $CD8^+CD122^+$ T cells in the liver of normal mice [7, 10]. Since the Fas (CD95) gene is muted in *lpr* mice [13], it may accelerate the proliferation instead of the apoptosis of activated $CD8^+CD122^+$ T cells in the liver, and they may migrate into periphery after downregulation of CD8 [10].

Bacteria and their components, lipopolysaccharide, peptidoglycan-polysaccharide, and various toxins are physiologically brought from the intestine to the liver [14, 15] and may stimulate these liver leukocytes and their antimicrobial and antitumor immune function. In addition, the IL-6 produced by Kupffer cells/hepatocytes stimulates hepatocytes to produce acute phase proteins (including CRP) and subsequent complement production [16–18]. Therefore, the liver is not only the organ for sugar, protein, and lipid/cholesterol metabolism but also an immune organ. This review focuses on the crucial role of the liver leukocytes in the antitumor and antimetastatic immunity.

2. Inhibition of Hematogenous Tumor Metastases in the Liver by NKT Cells Stimulated with Recombinant Interleukin-12 (IL-12)

IL-12 was discovered in both mice and humans around 1990 as an NK cell stimulatory factor [19–21]. IL-12 was initially thought to activate NK cells and cytotoxic $CD8^+$T cells to inhibit tumor metastasis. However, we found that the main effector cells that inhibit tumor metastasis of intravenously (i.v.) injected tumors are NKT cells [22–25]. When liver metastatic EL-4 cells (lymphoma), lung metastatic 3LL cells (Louis lung carcinoma), and other tumors were injected into B6 or other strains of mice via a tail vein, the main antimetastatic effectors in the liver, as well as in the lung, were NKT cells (Table 1) [22–25]. However, NK cells were not significantly involved, because IL-12 exerted a potent antimetastatic effect in the liver and lung in NK-deficient beige (bg/bg) mice (Table 1) [23]. In addition, the depletion of both NK cells and NKT cells by anti-NK1.1 Ab, but not the depletion of NK cells alone by an asialo-GM1 Ab, inhibited the IL-12-induced antimetastatic effects in both organs (Table 1) [25]. Furthermore, adoptive transfers of various sorted lymphocyte subsets in liver MNCs from IL-12-injected mice into tumor-inoculated mice confirmed that NKT cells, but not NK cells or $CD8^+$T cells, are antimetastatic effectors in the liver, the lungs, and kidneys [3, 24]. These results were further confirmed in NKT

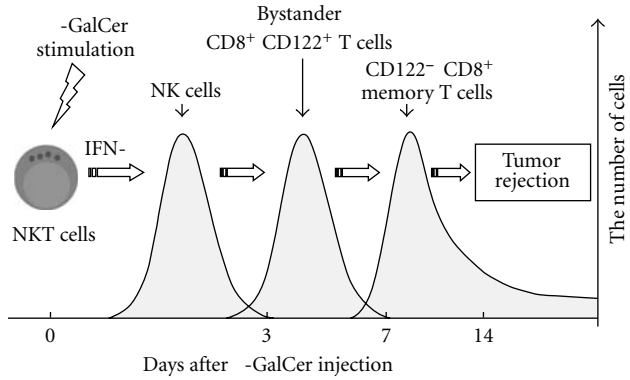


Figure 1: Sequential activation of liver lymphocytes and antitumor immunity by α-GlaCer.

cell-deficient mice [26]. However, NK cells and CD8+ T cells seem to be effectors against subcutaneous tumor growth [3].

Although some researchers have claimed that NKT cells disappear after IL-12 injection by activation-induced apoptosis, and therefore could not be the antimetastatic effectors, we demonstrated that IL-12 merely downregulates NK1.1 expression on NKT cells [27]. NKT cells in IL-12-pretreated mice (24 hours before) were further activated by the injection of a synthetic ligand, α-galactosylceramide (α-GalCer), and were observed to produce much more IFN-γ, as well as IL-4, and to acquire a more potent antitumor cytotoxicity than those in mice without IL-12 pretreatment [27]. It should be noted, however, that IL-12 pretreatment increased TNF receptor and Fas-ligand (FasL) of NKT cells and thereby augmented hepatotoxicity of NKT cells after α-GalCer injection [27]. However, as described hereinafter, such hepatotoxicity of α-GalCer-activated NKT cells can be completely inhibited by an anti-TNF-Ab without attenuating the antitumor immunity of the NK cells.

3. Inhibition of the Tumor Growth in the Liver by α-GalCer and Induction of Bystander CD8+ CD122+ T Cells and Tumor-Specific Cytotoxic CD8+ T Cells

α-GalCer was initially identified and extracted from a marine sponge, and thereafter synthesized by Kirin Brewery Company [28], and was subsequently observed to strongly inhibit the liver and lung growth of i.v. injected tumor cells. α-GalCer was found to be a ligand of the invariant Vα14Jα18/Vβ8 TCR of mouse NKT cells [29]. Therefore, NKT cells were initially thought to be antitumor effectors in the liver and lung, but NK cells were also suggested to be antitumor effectors after α-GalCer injection. However, the mice injected with α-GalCer were shown to have hepatic injury [30, 31]. Thereafter, we demonstrated that the NK cells stimulated with IFN-γ produced by α-GalCer-activated-NKT cells are the main antitumor effectors, whereas NKT cells themselves are not antitumor effectors, but they do induce hepatotoxicity as a result of their increased FasL expression [31, 32], in which lymphocyte infiltration and apoptotic hepatocytes (Councilman bodies) were observed in and around the portal areas. In addition, although NKT cells were initially thought to disappear due to apoptosis, and thus would not be able to further attack hepatocytes, it was subsequently found that the NKT cells merely transiently downregulated both NK1.1 and TCR [33, 34], in a manner similar to that observed after the injection of IL-12 [27]. These findings suggest that NKT cells downregulate their receptors to inhibit their autoreactivity.

The antitumor function of liver NK cells and the liver injury resulting from NKT cells induced by α-GalCer both increase age-dependently [31, 32]. Interestingly, however, when an anti-TNF Ab was injected simultaneously with α-GalCer into aged mice after i.v. injection of EL-4 tumor cells or intrasplenic injection of B16 tumor cells, the hepatic injury was completely inhibited, without attenuating the antitumor and antimetastatic activity of the liver NK cells [35].

The α-GalCer-induced NK cells with antitumor activity can kill not only NK-sensitive Yac-1 cells but also NK-resistant B16 cells, EL-4 cells and Colon 26 cells, and can inhibit the liver and lung metastasis of these NK-resistant tumors [36, 37]. Therefore, such activated NK cells may upregulate their killer activating molecules and/or downregulate their killer inhibitory molecules (e.g., CD94/NKG2A) as described in Section 4. Furthermore, after the activation of NK cells, bystander $CD8^+CD122^+TCR^{int}$ cells and tumor-specific memory CD8+T cells were induced after α-GalCer injection, thus allowing the mice to survive. Therefore, if such memory is achieved against certain tumors (e.g., B16 cells), these mice can reject subcutaneously rechallenged B16 cells but cannot reject other tumors (EL-4, Colon-26, etc.) [38]. Following NK cell activation for 2 to 3 days after α-GalCer injection, bystander CD8+CD122+cells with NK cell-like antitumor activity without tumor-specificity are increased at 3 to 7 days after α-GalCer injection, while memory CD8+T cells, which are cytotoxic only against certain tumors, are induced within two weeks (Figure 1).

Clinical trials using i.v. transfer of α-GalCer-pulsed DCs or PBMCs stimulated with α-GalCer in vitro for patients with advanced nonsmall cell lung cancer have been reported. In one report, α-GalCer-pulsed PBMCs cultured with IL-2 and GM-CSF were injected into patients four times, and the patients with increased IFN-γ producing cells in the PBMCs showed a longer survival (31.9 months, $n = 17$) than the poor responder patients (9.7 months, $n = 7$) [39]. Although no severe adverse event related to the treatment was observed, among several clinical trials, there was no case of obvious tumor regression [39], and a further evaluation of the survival benefit of such immunotherapy is required. It should also be noted that α-GalCer-reactive (specific) NKT cells are rare in humans as described in Section 5.

4. Antitumor Immunity in the Liver Induced by Bacterial Reagents

4.1. Lipopolysachharide (LPS). When mice were intraperitoneally (i.p.) or i.v. injected with a gram negative bacteria

component, LPS, Kupffer cells were activated via toll-like receptor- (TLR-) 4 [40] and produced IL-12, which stimulated NK cells to produce IFN-γ and activated NKT cells to acquire potent antitumor cytotoxicity [41]. As noted in Section 1, exogenous IL-12 injection stimulates the IFN-γ production and antitumor cytotoxicity of NKT cells, whereas NK cells are not main IFN-γ producers nor enhance their antitumor cytotoxicity. However, in the case of LPS injection, NK cells are the essential IFN-γ producers, while NKT cells are the main antitumor effectors [3]. This relationship between NK cells and NKT cells after LPS injection is opposite to that after α-GalCer injection (Table 2). Therefore, the IFN-γ-producing cells and final antitumor effectors differ based upon the stimulating reagent, whereas Kupffer cells are a constant provider of IL-12 [3].

The Kupffer cells activated by LPS also produce IL-6, which stimulates hepatocytes to produce acute phase proteins (including CRP) and complement components [3]. CRP stimulates Kupffer cells via Fcγ receptor II and enhances their phagocytic activity [42]. Since a small amount of LPS is considered to be continuously brought to the liver from the intestines via portal vein, such an environment in the liver induces a predominant presence of NK cells and NKT cells in the liver sinusoids [3]. In fact, when mice are maintained under the conventional condition, the number of liver MNCs, including NK cells, NKT cells, and $CD8^+$ $CD122^+$ T cells, are increased up to 2-fold compared to the numbers in mice maintained under SPF conditions, especially in aged mice [43]. Although LPS injection into mice triggers substantial antitumor immunity in the liver against liver metastatic tumors (EL-4 cells, etc.), in contrast to IL-12, LPS exerts antimetastatic effects only when injected before, but not after, tumor inoculation [41]. It is suggested that LPS, but not IL-12, induces potent TNF production from Kupffer cells/macrophages, which may induce adverse effects on the host defense, especially in tumor-inoculated mice. In fact, TNF reportedly increased tumor metastasis to the lungs [44].

4.2. Streptococcal Reagents. It has been well documented that when a *Streptococcus pyogenes* derivative (OK432) is injected to mice, the liver NK cells are increased and activated, and they suppress tumor metastasis in the liver [45, 46] (Table 2). T cells and NKT cells are not likely involved in this antitumor effect, because depletion of NK cells alone by an antiasialo GM1 Ab greatly diminished the antimetastatic effect of OK432. Since *Streptococcus pyogenes* is a gram positive bacteria that lacks LPS, either the teichoic acid, peptidoglycan-polysaccharide, or DNA motifs of *Streptococcus pyogenes* may stimulate Kupffer cells to produce IL-12 either through TLR-2 (teichoic acid, peptidoglycan-polysaccharide) or TLR-9 (bacterial DNA).

4.3. Bacteria DNA Motifs (CpG-ODN). CpG-ODN (oligodeoxynucleotides; GACGTT for mouse, GTCGTT for humans) has been shown to activate innate immunity via the TLR-9 expressed by macrophages [47–49]. This is an important finding, because these DNA motifs are common in all bacteria, and every bacterial infection or invasion can activate innate immunity in both humans and mice [49]. The differences in the frequency of unmethylated CpG dinucleotides between bacterial and vertebrate DNA provide a structural characteristic through which vertebrate immune cells are activated and respond to a bacterial infection [47, 49]. The CpG-ODN thus mimics the stimulatory effect of the DNA of either gram-negative of gram-positive bacteria. When CpG-ODN was injected into mice, the mouse Kupffer cells produced IL-12 and TNF and activated NK cells, as well as NKT cells in the liver (Figure 2).

Table 2: Antitumor or hepatotoxic effectors in the liver.

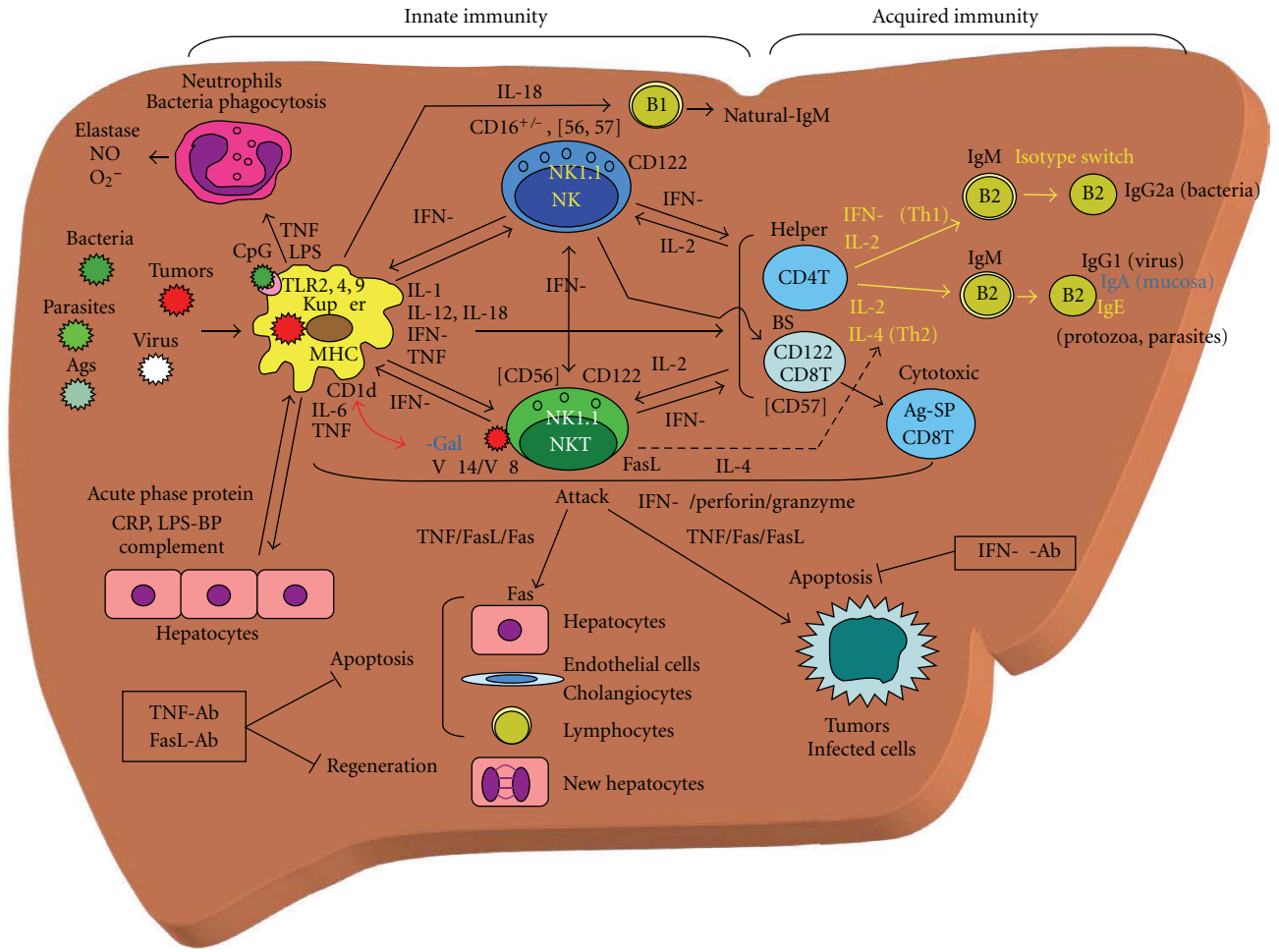
Function	Reagents				
	IL-12	α-GalCer	LPS	OK432	CpG
Antitumor effectors	NKT	NK	NKT	NK	NK
IFN-γ producers	NKT	NKT(NK)	NK	NK	NK
Hepatotoxic effectors	NKT	NKT	NKT/NK	?	NKT

Interestingly, IL-12-activated NK cells showed antitumor cytotoxicity after CpG-ODN injection, whereas NKT cells activated by TNF induced hepatocyte injury by expressing FasL [50]. Although the antitumor cytotoxicity and IFN-γ production of NK cells is attenuated with aging, the TNF production from Kupffer cells and FasL expression and hepatotoxicity of NKT cells are both augmented with aging [50]. The antitumor activity of CpG-ODN-stimulated NK cells may also be mediated by interferon-α [51], and the IFN-α production was also decreased with age [50]. Again, although the three bacterial reagents described above all activate Kupffer cells to produce IL-12, it is not clear at present why NKT cells are the main antitumor effectors induced by LPS, while NK cells are the main antitumor effectors induced by Streptococcal derivative and CpG-ODN (Table 2). A further study is needed to address this issue.

It should be noted that although several bacteria and their components have been suggested to be a natural ligand of NKT cells, we feel that certain bacteria or their components are not likely to be a ligand of NKT cells. As described above, activation of NK/NKT cells by LPS or CpG-ODN suggests that every gram positive or negative bacterium can indirectly activate NKT cells. Furthermore, major effectors to fight against bacteria are macrophages and neutrophils.

5. Antitumor Cytotoxicity of Human $CD56^+$T Cells, $CD16^+CD56^+$NK Cells, and $CD16^-CD56^{++}$ NK cells

It has been proposed that human NKT cells could be T cells bearing Vα24Jα18/Vβ11 gene products for their TCR, because their TCR genes show sequence homology with the mouse TCR Vα14Jα18/Vβ8 genes of NKT cells. In addition, both such T cells in mice and humans are specifically activated and proliferated by stimulation with α-GalCer. However, we demonstrated that Vα24Jα18/V$\beta11^+$ T cells are very rare in human peripheral blood and liver MNCs [3, 52].



Ag-SP: antigen-specific
BS: bystander
CRP: C-reactive protein
LPS-BP: LP binding protein
[]: in human

Figure 2: Scheme of immune responses in the liver.

Even in the liver MNCs, they occupy less than 0.5% of T cells, and we proposed that $CD56^+$ T cells (mostly $CD8^+$) are the human counterpart of mouse NKT cells. The reasons are as follows. (i) Human liver MNCs contain 25% $CD56^+$ NK cells and 20% $CD56^+$ T cells, similar to mouse liver NK cells and NKT cells [53]. (ii) The $CD56^+$ T cells vigorously proliferate and are activated after stimulation with IL-2 and IL-12 and acquire potent antitumor cytotoxicity [53, 54]. (iii) $CD56^+$ T cells have intermediate and pauciclonal TCRs similarly to mouse NKT cells [55]. The NKT cells and NK cells therefore likely play an important role in preventing tumor growth and metastases in the human liver as well as in mouse liver.

Most human NK cells in peripheral blood mononuclear cells (PBMCs) are $CD16^+CD56^+$ NK cells (10~15% of PBMC), while a small number of $CD16^-CD56^{++}$ NK cells, which express higher levels of CD56 than conventional $CD16^+CD56^+$ NK cells, are present (approximately 1% in PBMCs and 10% of NK cells) [56–58]. Although $CD16^-CD56^{++}$ NK cells are far less cytotoxic than $CD16^+CD56^+$ NK cells in their resting state, when purified and stimulated with IL-2, IL-12, and IL-15 for several days, the $CD16^-CD56^{++}$ NK cells proliferate more vigorously compared to $CD16^+CD56^+$ NK cells, and some $CD16^-CD56^{++}$ NK cells acquire CD16 expression. These $CD16^+$ $CD56^{++}$ NK cells produce a large amount of IFN-γ and display strong antitumor cytotoxicities against not only NK-sensitive K562 cells but also NK-resistant Raji cells [58–61]. These cells are also induced by Streptococcal derivative and heat-killed *Streptococcus* from PBMC or $CD16^-CD56^{++}$ NK cells [58]. Although most of these cells express NKG2A (an NK-inhibitory receptor), they also express NKG2D (an NK-activating receptor) and other natural cytotoxicity receptors (NKp30, NKp44, and NKp46) and therefore can kill NK-resistant tumors [58]. Interestingly, the majority of NK cells in the liver, colon, lymph nodes, uterus, and placenta are $CD16^-CD56^+$ NK cells [58, 62]. Therefore, these cells in the human liver, when activated, may have the potential to produce IFN-γ and kill various tumors. It can be speculated

that NK cells in PBMCs are moving in the rapid blood flow in vessels and can therefore monitor pathogens and tumor cells that invade the blood stream. They need to have the NK activity to immediately attack virus-infected cells and malignant cells and express CD16 (FcγRIII), presumably for induction of antibody-dependent cell-mediated cytotoxicity (ADCC) of infected cells, microbes, and tumors.

On the other hand, since NK cells in organs do not usually encounter pathogens, they do not need to be in an activated state. However, when once a pathogen/bacteria invaded the organs, they need to be activated to reject the pathogens. However, together with NKT cells, tissue macrophages, and neutrophils, these cells sometimes induce tissue damage and multiorgan dysfunction (MODS) as a result of their autoreactivity, as is the case in septic shock. Therefore, in order to reduce tissue damage, they are thought to normally be in resting states. It should be noted that mouse counterpart of human $CD16^{-}CD56^{++}$ NK cells cannot be identified because mouse NK cells do not express CD56. However, since activated NK cells induced by α-GalCer, CpG-ODN, or a Streptococcal derivative can kill NK-resistant tumors, similar NK cells may also exist in mice. Whether these $CD16^{-}CD56^{++}$ NK cells and $CD16^{+}$ $CD56^{+}$ cells with NK activity are the same lineage cells or distinct subsets needs further investigation.

6. $CD16^{-}CD56^{++}$ NK Cells in Diseases and in the Clinical Setting

As described perviously, $CD16^{-}CD56^{++}$ NK cells and their production of IFN-γ may play an important role in antitumor immunity; however, the expansion of $CD16^{-}CD56^{++}$ NK cells has been observed in some diseases and in the clinical setting. These cells are the first lymphocytes to appear in the PBMCs after bone marrow transplantation [63]. These cells are also reportedly expanded in the PBMCs of patients with systemic lupus [64], in the synovial fluid of patients with rheumatoid arthritis, and in patients with autoimmune hepatitis [65]. In addition, as described above, these cells were found to expand in vitro after stimulation of PBMCs with a *Streptococcus pyogenes* reagent (OK-432) [58], suggesting their involvement in bacterial infections. In contrast, the number of $CD56^{++}$ NK cells was decreased in the PBMCs of patients with allergic rhinitis and/or asthma [66], suggesting their role in Th1 but not Th2 immune responses.

It has recently been reported that liver $CD56^{+}$ NK cells (presumably $CD16^{-}$ cells) were increased in the livers of primary bilially cirrhosis (PBC) patients. These cells are frequently seen in the portal area, within the biliary epithelium, and around bile ducts [67]. NK cells from the PBC livers stimulated with a combination of TLR-4 and TLR-3 ligands (LPS and Poly I:C, resp.) in vitro exhibited a higher cytotoxic activity against autologous primary human biliary epithelial cells (cholangiocytes) than liver MNCs from subjects with other liver diseases (viral hepatitis and alcoholic liver disease), in which IFN-α-produced Kupffer cells stimulated by the TLR-3 ligand may also be required [67]. These findings suggest an important role for $CD56^{+}$ NK cells in PBC. Regarding NK and NKT cells in autoimmune diseases, it should be noted here that NKT cells in mice and humans reportedly inhibit autoimmune diseases (systemic lupus, experimental encephaolomyelitis, Type I diabetes, etc.). However, the role of NKT cells in autoimmune diseases should be carefully evaluated, because NKT cell activation by α-GalCer conversely accelerated the onset of lupus-like symptoms, autoantibody production, and hepatotoxicity in NZB/W mice [30, 68]. Further, the effect of α-GalCer depends on the mouse strains being examined [69].

Overall, these findings suggest that $CD16^{-}CD56^{++}$ NK cells, together with conventional NK cells, NKT cells, and Kupffer cells may play significant roles in Th1 immune responses against cancers and infections, in some autoimmune diseases, and also presumably in nonalcoholic steatohepatitis (NASH).

7. The Effects of Aging on Antitumor Immunity, Septic Shock, and MODS

The α-GalCer-induced antitumor immunity in the liver (antitumor cytotoxicity) produced by NK cells and the MODS induced by NKT cells unexpectedly both increases with age [32]. In general, antitumor immunity in the liver and other organs appears to decrease with aging, although the proportions of $CD57^{+}$ T cells (a human counterpart of mouse $CD8^{+}CD122^{+}$ TCR^{int} cells) and NK cells increase with aging [54]. Consistently, CpG-ODN-induced antitumor immunity and IFN-γ production from liver NK cells decreases age-dependently [50]. The septic shock and MODS in mice induced by CpG-ODN administration [70] also worsened age-dependently, because macrophages/Kupffer cells produce a large amount of TNF, and NKT cells increase their FasL expression [50]. The septic shock induced by IL-12 and low-dose LPS (16 h apart) is called the generalized Shwartzman reaction (GSR) and the GSR is also aggravated with aging, because $CD8^{+}CD122^{+}$ cells with IFN-γ producing capacity and the TNF production by macrophages/Kupffer cells (final effectors for MODS) both increase age-dependently [71]. Thus, liver innate immunity can be a double-edged sword.

Using human PBMCs, an in vitro GSR-like phenomenon can also be reproduced when the PBMCs are stimulated with IL-12 and LPS (24 h apart), because NK cells and $CD57^{+}$ T cells with IFN-γ producing capacity increase with age, and the TNF production from macrophages also increases with age [72]. These results explain why septic shock after abdominal surgery occurs more frequently in elderly patients [72]. Thus, innate immunity is a double-edged sword, and aging attenuates the antitumor anti-microbial immunity but aggravates tissue damage. Tissue damage or MODS can be avoided by the administration of an anti-TNF-Ab [35, 50], but the occurrence of any side effects (bacterial infection, especially tuberculosis) should be carefully monitored. In this regard, synthetic CRP may be an effective modulator of innate immunity, which enhances the phagocytic actvity of Kupffer cells and reduces their TNF production, without

attenuation of IFN-γ production from NK/NKT cells [42]. In fact, the administration of synthetic CRP improved the survival of the mice from bacterial infections and GSR [42].

8. The Role of Liver NKT Cells and NKT Cells in Hepatitis C Cirrhosis Patients and the Development of Hepatocellular Carcinoma (HCC)

We previously demonstrated in hepatitis C patients that NKT cells ($CD56^+$ T cells), and subsequently, $CD56^+$ NK cells, constantly decrease as hepatitis C progresses to cirrhosis, and most of NKT cells and NK cells are lost in cirrhotic livers [52]. Consequently, when liver MNCs obtained from surgical liver specimens of cirrhosis patients with HCC were cultured with IL-2, IL-12, and IL-15, they showed decreased IFN-γ production and antitumor cytotoxicity against both K562 cells and Raji cells, which was also the case against an HCC cell line (HuH-7 cells) [52]. Liver NK cells can kill MHC class-I (−) K562 cells, but not MHC class I (+) Raji cells, because MHC class-I molecules inhibit NK cell cytotoxicity by inhibitory signaling, while NKT cells effectively kill Raji cells, but not K562 cells. Interestingly, since HuH-7 cells express low levels of MHC class-I, cytokine-activated NK cells more effectively kill HuH-7 cells than NKT cells [52]. These results suggest that the decrease of NK cells, as well as NKT cells and their antitumor activities, is an important immunological mechanism that may allow the development of HCC in hepatitis C-associated cirrhotic livers. It was also reported in mice that NKT cells were lost in CCL4-induced cirrhotic livers [73]. These results suggest that maintenance of NKT cells in the liver requires normal organization of liver parenchyma. However, notably, if CD94/NKG2A (inhibitory receptors) were blocked by an antibody, NK cells could effectively kill MHC class-I (−) tumors [54]. In addition, since most liver NK cells are CD16 negative and can be activated by cytokines produced by Kupffer cells (IL-12, etc.) and may express NKG2D and other activating molecules, they can kill class-I (−) tumors. These findings suggest that the relationship between NK cells ($CD16^{+or-}$)/NKT cells and tumor cells in the liver during antitumor immunity is more complex than previously expected.

Although the functional impairment of NK cells and NKT cells may also play an important role in the development of HCC in hepatitis B patients, we could not find any decrease in $CD56^+$ T cells and NK cells in the livers of HCC patients with hepatitis B (our unpublished observation), suggesting that the behavior of lymphocytes in hepatitis C and hepatitis B may be different. It is known that, although most HCC cases develops in cirrhotic livers with hepatitis C, HCC also develop in livers with hepatitis B patients without apparent cirrhosis.

9. Possible Interactions of TNF, NKT Cells, and FasL with Hepatocytes

As described previously (Sections 3 and 4), although both α-GalCer and CpG-ODN induce antitumor activity by hepatic NK cells, they also activate NKT cells to induce hepatocyte injury through the TNF/FasL/Fas pathway [31, 32, 50]. In this regard, it has been unclear whether NKT cells express FasL only to damage hepatocytes, or whether there is a protective function. An important finding was that both α-GalCer and CpG-ODN induce hepatocyte injury in aged mice, but not in young mice [32, 50]. Furthermore, α-GalCer-activated NKT cells accelerate hepatocyte and liver regeneration after 70% partial hepatectomy (PHx) in mice, which is also TNF/FasL-dependent, whereas NK cells are inhibitory to liver regeneration [74]. In Fas-mutated autoimmune *lpr* mice and NKT cell-deficient CD1d−/− mice, and in normal B6 mice depleted of TNF or FasL by neutralizing Abs, there was no accelerated regeneration of the PHx liver after α-GalCer injection [74] (Figure 2). Consistent with these results, it was reported that injection of exogenous TNF or anti-Fas Ab into PHx mice accelerated the regeneration of the PHx liver [75–77]. These findings suggest that NKT cells may normally regulate the turnover of hepatocytes (newly generated hepatocytes and old hepatocytes), the normal lifespan of which is around 200 days [78]. Hepatocytes nascent at the portal space gradually stream toward the terminal hepatic vein, where they are probably eliminated by apoptosis [78]. However, since most HCC shows reduced Fas expression in both hepatitis B and C patients [79–81], HCC may develop by evading surveillance of FasL-expressing NKT cells.

10. The Role of NK Cells, NKT Cells, and Kupffer Cells in the Development of Liver Metastasis of Colon Cancers

Malignant tumors, especially those of the colon and stomach, metastasize to the liver via the portal vein. Several experimental studies in mice and rats have demonstrated that NK cells are important antimetastatic effectors in the liver. NK cells are located in the liver sinusoids and adhere to sinusoidal endothelial cells and Kupffer cells, which bind to colon tumor cells injected from mesenteric veins, and kill them. Since anti-asialoGM1 Ab treatment of mice, which specifically depletes NK cells, greatly increased the number of metastases of colon cancers, NK cells were considered to be the main antimetastatic effectors [46]. Interestingly, when a OK432 was injected i.v. into mice, NK cells increased in the liver, and the antimetastatic function of the liver MNCs against colon cancers greatly increased [46]. This was also the case for α-GalCer and liver NK cells. However, as described above, the administration of either IL-12 or LPS activates NKT cells and inhibits tumor metastasis in the liver. Therefore, NK cells and NKT cells either independently or cooperatively act as antitumor effectors both in mice and humans. However, the antitumor effects of Kupffer cells themselves are controversial. Although the cytokines produced by Kupffer cells (IL-12, IFN-α) are indeed important for the activation of NK cells and NKT cells and for preventing tumor liver metastases, depletion of Kupffer cells by gadolinium chloride or clodronate liposomes increased the number of liver metastasis in some reports [82, 83] while

it did not affect the number of tumor metastases in the liver in other reports [46]. In vitro experiments also showed that Kupffer cells can phagocytose tumor cells and can kill them [84], although another report contradicted this claim [85].

Our unpublished observations showed that NK cells and NKT cells in the human liver tissues close to metastatic colon tumors express less perforin than those in the liver tissues distant from metastatic tumors, implying that tumor metastasis starts to grow at the area where lymphocyte activity is attenuated. Alternatively, tumors may produce paracrine factors which may inhibit perforin production and antitumor cytotoxicity of NK/NKT cells around tumors.

11. Concluding Remarks

The liver contains innate immune effectors, Kupffer cells, NK cells, NKT cells, and $CD8^+CD122^+$ cells, and these cells cooperatively act not only against bacterial and viral infections but also against cancers. Many bacterial components and toxins from the portal vein and systemic circulation activate Kupffer cells to produce IL-12 and induce potent antitumor activity by NK cells, NKT cells, and $CD8^+CD122^+$ cells via IFN-γ/perforin/granzyme pathway (Figure 2). $CD16^-CD56^{++}$ NK cells in PBMC and presumably in the liver of humans may also play an important role in antitumor immunity, infections, and some autoimmune diseases. The IFN-γ produced by these innate immune lymphocytes in the liver in turn stimulates the phagocytic activity and cytokine production of Kupffer cells via a positive feedback loop (Figure 2). Liver NK cells, NKT cells, and $CD8^+CD122^+$ cells may also migrate to other organs to inhibit tumor growth there. Decreased NKT cells and NK cells in cirrhotic livers in hepatitis C patients may therefore allow for the development of HCC. However, the TNF produced by Kupffer cells and TNF-activated liver lymphocytes, NKT cells and NK cells, may be responsible for septic shock, hepatocyte injury/regeneration, cholangiocyte injury, and MODS via the TNF/FasL/Fas pathway (Figure 2).

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Research Article

HLA Class II Defects in Burkitt Lymphoma: Bryostatin-1-Induced 17 kDa Protein Restores CD4+ T-Cell Recognition

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While the defects in HLA class I-mediated Ag presentation by Burkitt lymphoma (BL) have been well documented, CD4+ T-cells are also poorly stimulated by HLA class II Ag presentation, and the reasons underlying this defect(s) have not yet been fully resolved. Here, we show that BL cells are deficient in their ability to optimally stimulate CD4+ T cells via the HLA class II pathway. The observed defect was not associated with low levels of BL-expressed costimulatory molecules, as addition of external co-stimulation failed to result in BL-mediated CD4+ T-cell activation. We further demonstrate that BL cells express the components of the class II pathway, and the defect was not caused by faulty Ag/class II interaction, because antigenic peptides bound with measurable affinity to BL-associated class II molecules. Treatment of BL with broystatin-1, a potent modulator of protein kinase C, led to significant improvement of functional class II Ag presentation in BL. The restoration of immune recognition appeared to be linked with an increased expression of a 17 kDa peptidylprolyl-like protein. These results demonstrate the presence of a specific defect in HLA class II-mediated Ag presentation in BL and reveal that treatment with bryostatin-1 could lead to enhanced immunogenicity.

1. Introduction

Burkitt lymphoma (BL) is an aggressive non-Hodgkin's B-cell malignancy, occurring most frequently as endemic BL in children living in areas of high malarial prevalence [1]. This malignancy may also be found in other parts of the world as sporadic BL and accounts for 1-2% of all lymphomas in Western countries [1]. The clinical manifestations of BL are variable, with tumors of the jaw characteristically seen in endemic BL and tumors in the gut associated with sporadic BL [2–4]. BL has one of the fastest doubling times among human malignancies and is frequently associated with immune deficiency [3].

In addition to its strong association with malaria, BL has a high correlation with Epstein-Barr Virus (EBV). EBV infection, however, is not requisite for the development of BL, and the degree of association with EBV varies based on the type of BL. EBV infection occurs in >90% of endemic BL cases, 10–15% of sporadic BL, and 40% of human immunodeficiency virus (HIV) associated BL [1]. While the exact role that EBV plays in the development of BL remains largely unknown, it is understood that EBV gene products may be involved in the transformation of BL cells and their decreased immunogenicity. Additional evidence for EBV having a role in development of BL stems from EBV's link to various other lymphoid malignancies including

Hodgkin's lymphoma, transplant-related B-cell lymphomas, T-cell lymphomas, adult T-cell leukemia, and natural killer cell leukemia [5–8]. While BL has varying associations with malaria and EBV and in some cases is not associated with either, the one feature shared by all BLs is overexpression of the oncogenic transcription factor *c-myc*, which has a gene network comprising up to 15% of all known genes [9]. This abnormality results from the translocation of the *MYC* gene to an immunoglobulin locus leading to its constitutive activation [10–12].

BL is known to be deficient in HLA class I-mediated antigen (Ag) presentation to CD8+ T lymphocytes [13–15]. However, the role of HLA class II-mediated Ag presentation in generating an immune response to BL has not been fully elucidated. The class I defect has been well studied and is understood to result from the weak immunogenicity of EBV nuclear Ag 1 (EBNA1), which is poorly processed and presented through the class I pathway [16–18]. Another EBV gene product, gp42, has a role in mediating virus binding through interaction with HLA class II and it has been speculated to block the interaction between class II and the T-cell receptor [19, 20]. Although HLA class I-mediated activation of CD8+ T cells leads to Ag-specific lysis of tumor cells, an HLA class II response is vital for the generation of sustained immune responses [21]. Our laboratory has previously shown that B-cell lymphomas are deficient in HLA class II-mediated Ag presentation [22], and in this study we explore the role of B-cell-associated molecules in restoration of CD4+ T-cell recognition of BL cells.

The study presented here suggests that multiple defects may contribute to BL's inability to efficiently present Ag via HLA class II molecules. We confirm expression of a transfected HLA class II allele in both BL cells and EBV-immortalized B-lymphoblastoid cells (B-LCL), and demonstrate that the transfected HLA class II efficiently binds exogenously delivered Ag to form class II peptide complexes. However, while B-LCL were capable of CD4+ T-cell stimulation, BL cells were deficient in their ability to do so, and addition of external co-stimulation was insufficient to overcome this defect. In addition, treatment of BL cells with bryostatin-1 partially restored class II-mediated Ag presentation. This restoration was linked to the upregulation of a 17 kDa protein in bryostatin-treated BL which was expressed at low levels in untreated BL but highly expressed in B-LCL, suggesting that this protein may play a role in enhancing class II-mediated Ag presentation. In other studies, bryostatin-1 has been shown to increase HLA class II expression in dendritic cells and in a colorectal carcinoma cell line, but its effect on HLA class II expression and Ag presentation in lymphoid malignancies has not previously been evaluated [23, 24]. On the whole, these results suggest that BL possesses multiple defects which lead to an impaired ability to stimulate CD4+ T cells through HLA class II Ag presentation. These defects may provide the opportunity to develop novel immunotherapies leading to more targeted treatment of BL and other lymphoid malignancies. This study also provides a rationale for the further evaluation of bryostatin-1 as a therapeutic treatment of lymphoid malignancies.

2. Materials and Methods

2.1. Cell Lines. Human BL cell lines, Nalm-6, Ramos, and Ous, were maintained in complete RPMI-1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 50 U/mL penicillin 50 μg/mL streptomycin, and 1% L-glutamine (Mediatech, Manassas, VA). The Ous cell line was a gift from Dr. Christian Munz (Rockefeller University). The human B-lymphoblastoid cell lines (B-LCL) 6.16 and Frev were maintained in IMDM supplemented with 10% bovine growth serum (Hyclone, Logan, UT), 50 U/mL penicillin 50 μg/mL streptomycin, and 1% L-glutamine (Mediatech). Nalm-6, Ramos, and 6.16 cells were retrovirally transfected for constitutive expression of HLA-DR4 (DRB1*0401) with linked drug selection markers for hygromycin and histidinol resistance to generate Nalm-6.DR4, Ramos.DR4, and 6.16.DR4 [22, 25]. Frev did not require transfection of the class II allele as it constitutively expresses HLA-DR4. Surface HLA-DR4 expression in the transfectants was confirmed by flow cytometric analysis using the DR4-specific mAb, 359-F10 [22, 26, 27]. 6.16.DR4 cells were further transfected with DMα and DMβ for constitutive expression of HLA-DM molecules to generate 6.16.DR4.DM [22]. The expression of HLA-DM on 6.16.DR4.DM cells was confirmed by western blotting. T-cell hybridomas 2.18a and 1.21 recognize Ig κ residues 188–203 and 145–159, respectively, and were generated by immunization of DR4-transgenic mice as described [25, 28]. The T-cell hybridoma 17.9 (generously provided by D. Zaller, Merck Research Laboratories, Rahway, NJ) responds to human serum albumin (HSA) residue 64–76 K [29]. These T cell hybridomas are less dependent on co-stimulatory signals for their stimulation. Cells were cultured in RPMI 1640 with 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 50 μM β-mercaptoethanol (Invitrogen).

2.2. Antigens, Peptides, and Other Reagents. Human serum albumin (HSA) and human IgG *kappa* (IgG κ) were purchased from Sigma (St. Louis, MO). $HSA_{64–76\,K}$ peptide (sequence: VKLVNEVTEFAKTK) human IgG immunodominant peptide $\kappa_{188–203}$ (κI; sequence: KHKVYACEVTHQGLSS), and subdominant peptide $\kappa_{145–159}$ (κII; sequence: KVQWKVDNALQSGNS) were produced using Fmoc technology and an Applied Biosystems Synthesizer as described, dissolved in PBS, and stored at -20°C until used [25, 29, 30]. Reverse phase HPLC purification and mass spectrometry were used to analyze the peptide and showed a peptide purity >99%. Bryostatin-1 was purchased from Sigma.

2.3. Antigen Presentation Assays. B-LCL and BL were incubated with 0 μM, 5 μM, 10 μM, or 20 μM HSA Ag or HSA synthetic peptide for 3–24 h at 37°C in the appropriate cell culture media to determine optimal antigen concentrations to use in antigen presentation assays [22, 25]. Following titration, the same assays were carried out using only the optimal concentration of each antigen. Cells were then washed and co-cultured with the T-cell hybridoma 17.9 for 24 h at 37°C. In parallel assays, 2.18a and 1.21 were

stimulated with anti-CD3/CD28 prior to co-culture with Nalm-6.DR4, Ramos.DR4, or 6.16.DR4.DM which had been incubated with κI or κII [29]. Following co-culture, T cell production of IL-2 was quantitated by ELISA [31]. Assays were repeated in triplicate with standard error for triplicate samples within a single experiment being reported.

2.4. Western Blotting. Western blot analysis was performed on whole cell lysates of Frev, Nalm-6.DR4, 6.16.DR4.DM, and Ramos.DR4. Expression of HLA class II, Ii, and HLA-DM was analyzed as described previously [32, 33]. Densitometry was performed using a ChemiDoc XRS station (Bio-Rad) where the protein bands were analyzed using the Quantity One 4.6.3 software (Bio-Rad). Relative protein expression levels were stated as a ratio of specific proteins expressed/β-actin for each sample. Data are representative of at least three separate experiments.

2.5. IL-2 ELISA. IL-2 levels in Ag presentation assay supernatant were quantitated by ELISA. A 96-well ELISA plate was coated overnight at 4°C with purified rat anti-mouse IL-2 (Sigma). The plate was then washed and blocked with 2% BSA at RT for 30 m. After washing, standards and samples were plated in appropriate wells and incubated at RT for 2 h. A standard curve was generated using recombinant IL-2 purchased from R&D (Minneapolis, MN). The plate was washed, and biotinylated rat anti-mouse IL-2 (Sigma) was added and incubated at RT for 1 h. Following washing, avidin peroxidase (Pierce, Rockford, IL) was added to each well and incubated at RT for 30 m. The plate was washed, and PNPP substrate (Thermo Scientific, Rockford, IL) was added to each well and incubated at RT. Readings were taken every 30 m at 405 nm. IL-2 levels in sample wells are expressed in pg/mL, calculated from the standard curve. Assays were repeated in triplicate and expressed as mean IL-2 ± SEM.

2.6. Peptide Binding Assays. Nalm-6.DR4, Ramos.DR4, 6.16.DR4.DM, and Frev cells were fixed in 1% paraformaldehyde and then incubated overnight with 0 μM, 10 μM, or 20 μM biotinylated HSA peptide (b-HSA) in 150 mM CPB (pH 7.4), washed with PBS, and lysed on ice for 20 min with 50 mM Tris buffer (pH 8) containing 0.15 M NaCl and 0.5% IGEPAL CA 630 (Sigma) as described [30, 34]. Cell supernatants were added to plates (Costar, Cambridge, MA) previously coated overnight with the anti-human class II antibody 37.1 (kindly provided by L. Wicker, Merck Research Lab, Rahway, NJ). The captured class II-peptide complexes were detected with europium-labeled streptavidin (Pharmacia Fine Chemicals, Piscataway, NJ) using a fluorescence plate reader (Delfia, Wallac, Turku, Finland). The number of total DR molecules within B-LCL/BL cells was quantitated as described [28].

2.7. Bryostatin-1 Treatment of BL Cells. Nalm-6.DR4 and Ramos.DR4 were treated with 0, 20, or 40 nM of bryostatin-1 for overnight. Following incubation, untreated and bryostatin-treated cells were used in Ag presentation assays with HSA peptide followed by ELISA IL-2 quantitation as already described. In separate assays, Ramos.DR4 were treated with 40 nM bryostatin overnight and then fixed in 1% paraformaldehyde for 5 min. Following fixation, the cells were washed and incubated with 0 μM, 10 μM, or 40 μM of b-HSA for 3 h at 37°C with shaking. The cells were then washed and lysed in Hanks Balanced Salt Solution with 1% Triton-X-100 and protease inhibitors (PMSF and TLCK). The lysate was added to wells of a 96-well plate precoated with the anti-HLA class II antibody, 37.1. Captured class II peptide complexes were detected using streptavidin peroxidase and BD OptEIA TMB substrate reagents (BD, San Diego, CA). The reaction was stopped using 1 M phosphoric acid and the resulting absorbance was read at 450 nM.

Additionally, surface protein expression in 6.16.DR4. DM, untreated Nalm-6.DR4, and bryostatin-treated Nalm-6.DR4 was evaluated by SDS-PAGE protein separation. Nalm-6.DR4 cells were treated overnight with 40 nM of bryostatin-1. Following incubation, 6.16.DR4.DM, untreated Nalm-6.DR4, and bryostatin-treated Nalm-6.DR4 were washed in citrate phosphate buffer (CPB) to elute cell surface proteins. The resulting eluate was then subjected to SDS-PAGE. A 17 kDa band was excised from these gels and analyzed by MALDI TOF-TOF mass spectrometry. Proteins in CPB eluate from 6.16.DR4.DM were separated by electrophoresis on large, nonreducing gels. The 17 kDa band was excised and the protein was extracted by sonication in PBS on ice. Ramos.DR4 cells were incubated with the HSA peptide in the 17 kDa extract for use in Ag presentation assays as described. T cell production of IL-2 was quantitated [31].

2.8. Protein Extraction and Digestion. CPB eluate was obtained from 6.16.DR4.DM, untreated Nalm-6.DR4, and bryostatin-treated Nalm-6.DR4 as described previously [22]. Extracts were concentrated, and protein concentrations were measured, then run on a non-reducing gel, and stained with Coomassie blue. Gel plugs were excised and placed in an eppendorf tube. Each plug was washed with 50 mM ammonium bicarbonate for 10 minutes. Next, the plugs were destained using 25 mM ammonium bicarbonate in 50% acetonitrile for 15 minutes. The plugs were dehydrated with 100% acetonitrile for 15 minutes and dried in a speedvac. Each gel plug was covered with Proteomics Grade Trypsin (Sigma) and incubated at 37°C overnight. The supernatant was collected in a clean dry eppendorf tube. Peptides were further extracted with 1 wash of 25 mM ammonium bicarbonate for 20 minutes and three washes of 5% formic acid, 50% acetonitrile for 20 minutes each. The supernatant was collected and pooled after each wash then dried down in a speedvac to ~1 uL. Prior to analysis, the samples were reconstituted with 10 uL of 0.1% trifluoroacetic acid. Samples were then concentrated with a C18 Ziptip (Millipore) and eluted with 0.1% TFA, 50% acetonitrile, and 7.0 mg/mL α-cyano-4-hydroxycinnamic acid directly onto the MALDI target.

2.9. Mass Spectrometric Analysis (MALDI TOF/TOF). After the spots were dried completely, the plate was loaded into the

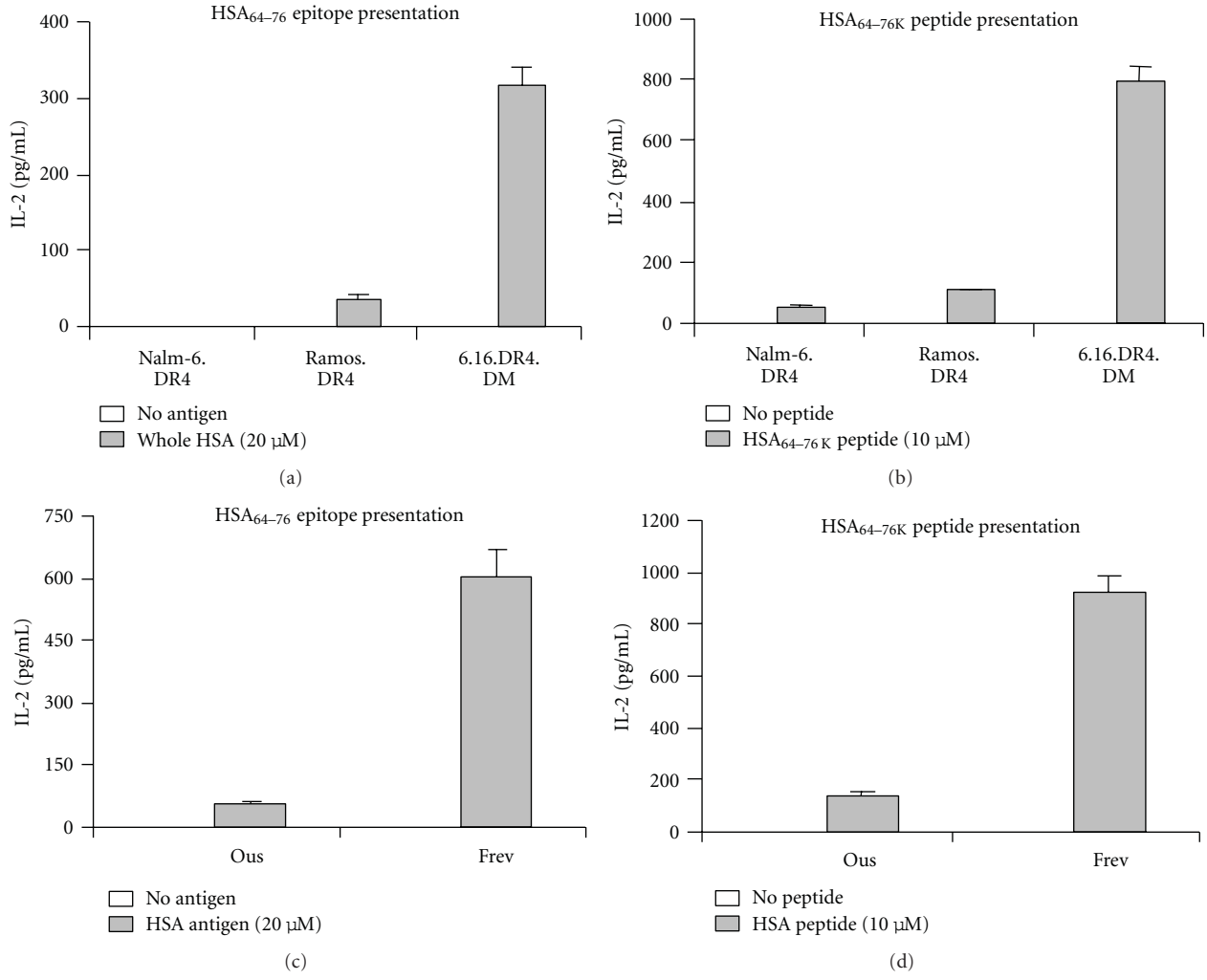
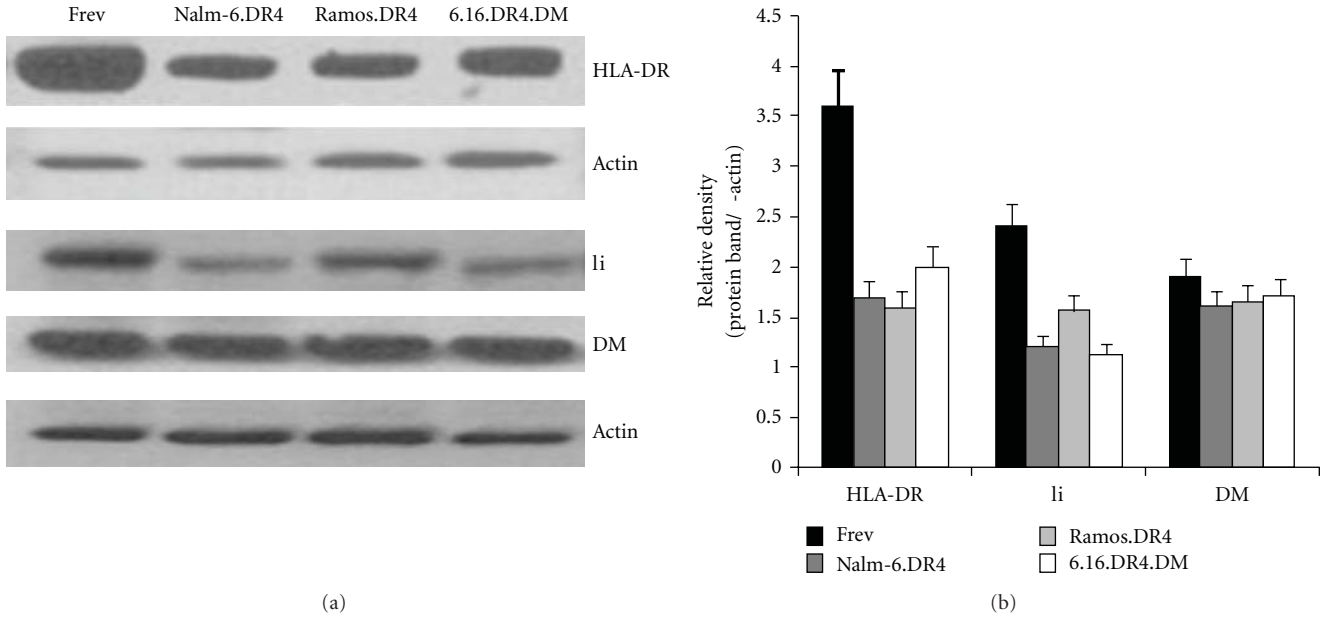


Figure 1: BLs are deficient in their ability to present Ag in the context of HLA class II. BL (Nalm-6.DR4, Ramos.DR4, and OUS) and B-LCL (6.16.DR4.DM and Frev) were incubated with whole HSA (a and c) or HSA synthetic peptide (b and d). Following incubation, cells were washed and co-cultured with the $HSA_{64–76\,k}$ epitope-specific T-cell hybridoma 17.9. Supernatant from the co-culture was assayed by ELISA to determine IL-2 levels as a measure of T-cell stimulation. All three BL cell lines were deficient in stimulation of IL-2 production for both whole HSA and HSA synthetic peptide, while both B-LCL efficiently presented each Ag to stimulate high levels of IL-2 production.

Applied Biosystems 4800 Proteomics Analyzer. An external calibration was performed prior to analyzing samples utilizing the manufacturer's standards and protocols. Samples were analyzed in batch mode using 2000 laser shots per spectrum. First, peptide mass maps were acquired over the m/z range of 800–3500 in reflectron mode with a delayed extraction time optimized for m/z 2000 by averaging 2000 scans to locate peaks of peptide origin. The next batch run performed MS-MS analyses to obtain sequence data on the 20 most abundant peaks from the MS analysis. Upon completion of the batch processing, the data was exported into the GPS Explorer data processing system for interpretation and identification. The MASCOT database-searching algorithm analyzed the data and summarized the results in report format. Database searches were performed using 2 missed cleavages and one differential modification of methionine oxidation. The top 20 matches were reviewed prior to assigning confident protein identifications.

3. Results

3.1. BL Cells Display Decreased HLA Class II-Mediated CD4+ T-Cell Stimulation. Although BL and B-LCL both express surface HLA class II, we transfected these cell lines to express a common class II allele so that we might obtain a more direct comparison of class II-mediated Ag presentation between the two cell types. Retroviral gene transfections of a DR4 allele, HLA DRB1*0401, were carried out on our BL (Nalm-6 and Ramos) and B-LCL (6.16) cell lines. Flow cytometric analysis confirmed transfection and expression of this allele in all three cell lines (data not shown). 6.16.DR4 cells were additionally transfected with HLA-DM to generate 6.16.DR4.DM cells expressing similar levels of HLA-DM when compared to Nalm-6.DR4 and Ramos.DR4 [33]. Transfectants were then sorted, matched for surface DR4 expression, and incubated, along with Ous and Frev, in culture media with either HSA antigen or HSA peptide. Following incubation, cells were



(a) (b)

Figure 2: BL and B-LCL express similar levels of HLA class II pathway components. Variations in efficiency of Ag presentation between BL and B-LCL may be attributable to differences in expression levels of components in the class II pathway. To evaluate this possibility, the BL cell lines Nalm-6.DR4 and Ramos.DR4, the B-LCL cell lines 6.16.DR4.DM and Frev were analyzed by western blotting for expression of HLA class II, invariant chain (Ii), and HLA-DM (a). Densitometric analysis confirmed the expression of comparable levels of each of these class II pathway components (b).

washed and co-cultured with the T-cell hybridoma, 17.9, for 24 h at 37°C. Culture supernatant was collected and assayed by ELISA for IL-2 levels. The results of these assays demonstrate that Nalm-6.DR4, Ramos.DR4, and Ous were deficient in their ability to stimulate IL-2 production in 17.9 by class II-mediated presentation of HSA epitope or HSA synthetic peptide (Figures 1(a)–1(d)). The B-LCL lines 6.16.DR4.DM and Frev, however, stimulated production of high levels of IL-2 (Figures 1(a)–1(d)). Supplemental Figure 1 (see Supplemental material available online at doi:10.1155/2011/585893) shows the results of whole HSA and HSA peptide titration with Nalm-6.DR4, Ramos.DR4 and 6.16.DR4.DM. Nalm-6.DR4 and Ramos.DR4 fail to stimulate IL-2 production at all concentrations of whole HSA or HSA peptide, while 6.16.DR4.DM shows a dose-dependent increase in levels of IL-2 production. These results suggest that BL cells possess a defect(s) in the presentation of Ag to CD4+ T cells in the context of HLA class II.

3.2. BL and B-LCL Express Similar Levels of HLA Class II Pathway Components. Western blot analysis was performed on Nalm-6.DR4, Ramos.DR4, 6.16.DR4.DM, and Frev for expression of HLA class II, Ii, and HLA-DM. Data from these analyses revealed that both BL and B-LCL expressed detectable levels of these immune components (Figure 2(a)). As a wild type B-LCL, Frev expresses higher levels of class II pathway components than Nalm-6.DR4, Ramos.DR4, and 6.16.DR4.DM, as analyzed by densitometry and corrected for actin loading controls (Figure 2(b)). These data suggest that the observed defect in class II-mediated Ag presentation by BL is not the result of a defect in the HLA class II processing and presentation pathway.

3.3. Addition of External Co-Stimulation Is Insufficient to Overcome the BL-Associated Defect in Class II-Mediated Ag Presentation. It has previously been reported that BL cells are deficient in expression of co-stimulatory molecules (CD80/86). In order to determine if this was the cause of the defect in class II-mediated Ag presentation by BL, Ag presentation assays were performed in the presence of external co-stimulatory signals as described. In this assay, T-cell hybridomas were treated with anti-CD3/CD28 plus cross-linked IgG and co-cultured with the BL cells that were preincubated with HSA peptide. The addition of external co-stimulation had little to no effect on class II Ag presentation by BL (Figure 3). While 6.16.DR4.DM stimulated high levels of IL-2 production in T cells with or without external co-stimulation, Nalm-6.DR4 and Ramos.DR4 showed no significant increase in stimulation of IL-2 production in T cells with external co-stimulation.

3.4. HSA Peptide Binds with Similar Affinity to HLA Class II on BL and B-LCL. The next step in evaluating the BL-related defect in HLA class II Ag presentation was to assess the binding efficiency of HSA peptide to BL-expressed surface DR4. Nalm-6.DR4, Ramos.DR4, 6.16.DR4.DM, and Frev were incubated with various concentrations of b-HSA at pH 7.4. Class II peptide complexes were then detected in an ELISA format using europium-labeled streptavidin. Data showed a dose-dependent response with each cell line binding b-HSA peptide with a similar, measurable affinity (Figure 4). These results suggest that BL's reduced capacity to present Ag via HLA class II is not a result of impaired peptide binding to HLA class II molecules.

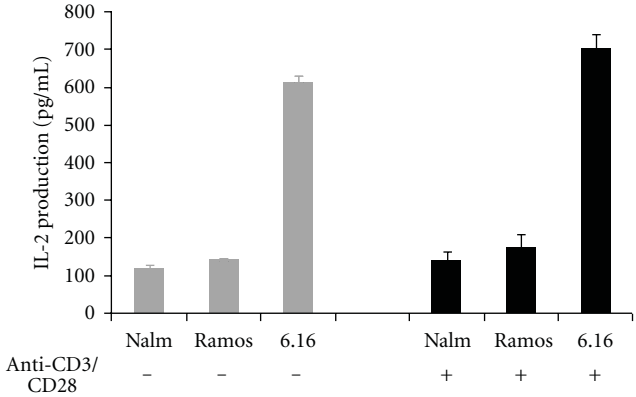


Figure 3: Addition of external co-stimulation is not sufficient to overcome class II-associated defects in BL. BLs are known to express lower levels of costimulatory molecules, raising the possibility that this was the cause of the observed defect in their ability to present Ag via class II. BL cell lines Nalm-6.DR4 and Ramos.DR4, and the B-LCL cell line 6.16.DR4.DM were incubated with κI and κII peptides prior to co-culture with the T-cell hybridomas 2.18a or 1.21 which had been stimulated with anti-CD3/CD28. Culture supernatant was assayed by ELISA for IL-2 levels as a measure of T-cell stimulation. These results demonstrate that Nalm-6.DR4 and Ramos.DR4 Ag presentation is unaffected by the addition of external co-stimulation and remains deficient in class II-mediated presentation.

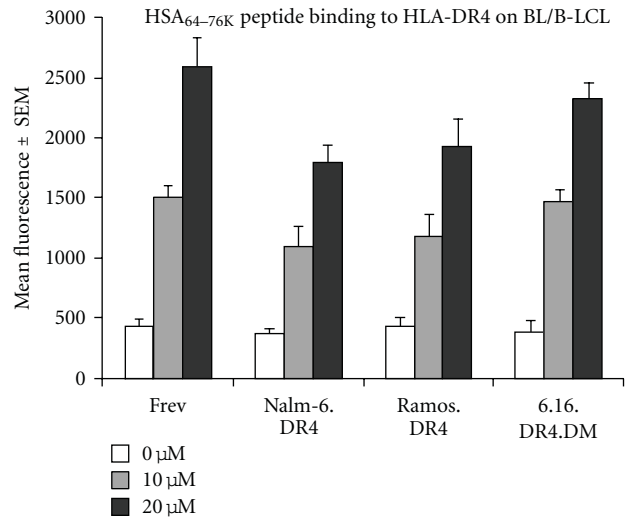


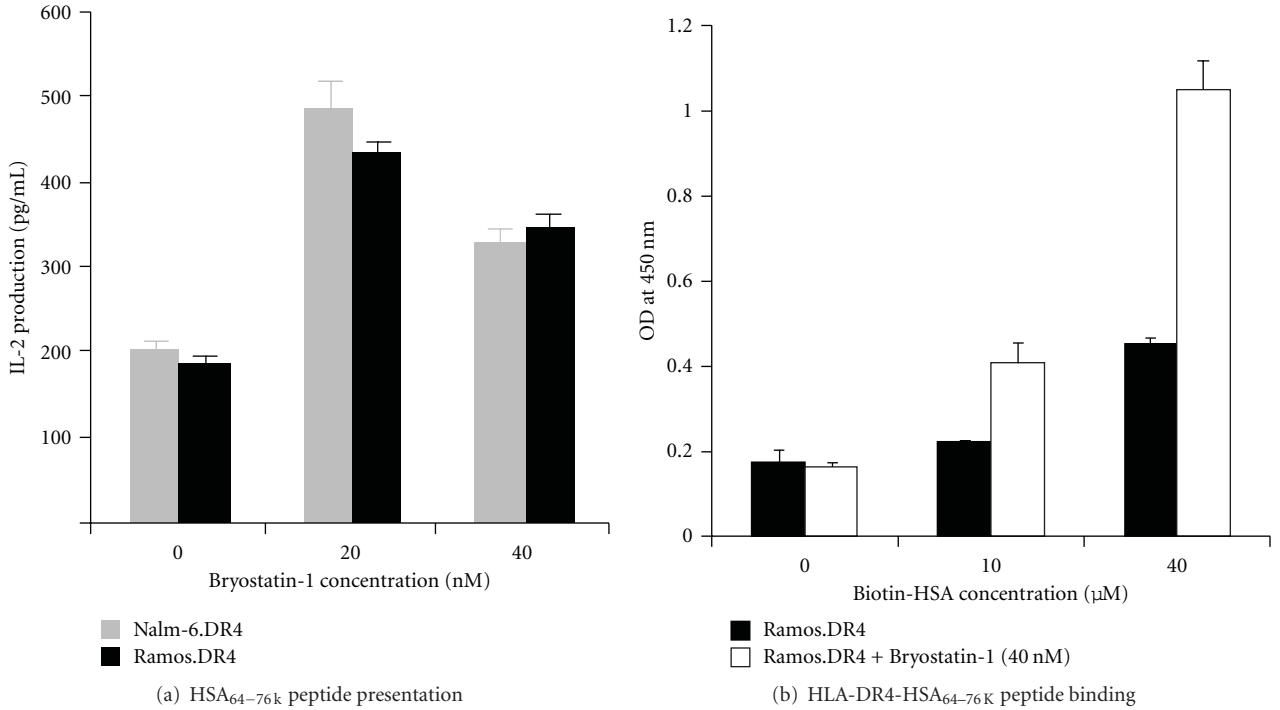
Figure 4: BL and B-LCL bind $HSA_{64–76K}$ synthetic peptide with similar affinity. Ag presentation depends on efficient binding of Ag to HLA class II proteins. BL cell lines Nalm-6.DR4 and Ramos.DR4, and the B-LCL lines 6.16.DR4.DM and Frev were fixed and incubated with biotin-labeled $HSA_{64–76K}$ at pH 7.4. Cells were lysed and class II peptide complexes were detected in ELISA format with europium-labeled streptavidin with mean fluorescence used as a measure of peptide binding. Each cell line bound similar levels of peptide.

3.5. Bryostatin Treatment of BL Increases Peptide Binding to HLA Class II and Restores Class II Ag Presentation and CD4+ T-Cell Recognition. Previous studies on bryostatin-1 have shown that it causes upregulation of HLA class II molecules in the professional Ag presenting dendritic cells and leads to increased T-cell stimulation by these cells [23]. Based on this finding, we sought to determine if bryostatin-1 treatment would impact Ag presentation by BL. Nalm-6.DR4 and Ramos.DR4 cells were treated with bryostatin-1 overnight and then used in Ag presentation assays with HSA as already described. Untreated BL cells showed similarly low levels of T-cell stimulation, whereas cells treated with bryostatin-1 at 20–40 nM restored Ag presentation and T-cell stimulation (Figure 5(a)). Ramos.DR4 cells were treated with bryostatin-1 overnight, and peptide binding to HLA class II was measured as already described. Ramos.DR4 treated with 40 nM bryostatin-1 showed significantly higher levels of peptide binding at both 10 μM and 40 μM b-HSA (Figure 5(b)).

3.6. Bryostatin Treatment Upregulates Expression of an Immunostimulatory 17 kDa Protein in BL. To determine the nature of the class II presentation restoration in BL following bryostatin-1 treatment, protein expression patterns in CPB eluates from Nalm-6.DR4 and 6.16.DR4.DM were analyzed by gel electrophoresis (non-reducing gel) and coomassie blue staining. This study showed that a 17 kDa protein was consistently expressed at low levels in BL cells (Nalm-6.DR4) but high levels in B-LCL (6.16.DR4.DM) cells (Figure 6(a)). Following overnight treatment of BL cells with bryostatin-1, expression of this 17 kDa protein was restored to levels comparable to 6.16.DR4.DM cells (Figure 6(a)). This protein band was then cut from the gel and analyzed by MALDI TOF/TOF mass spectrometry, revealing a peptidylprolyl-like protein (accession number: 89058151). To further analyze the function of this 17 kDa protein, CPB eluates from 6.16.DR4.DM cells or bryostatin-1-treated BL cells were separated on a large non-reducing gel, the band corresponding to 17 kDa protein was excised, and the protein was extracted by sonication in PBS on ice. Ramos.DR4 cells were then incubated with HSA peptide (10 μM) in the presence of this extract, followed by washing and co-culture with 17.9 T cells. ELISA IL-2 quantitation of the assay supernatant showed a significant increase in the stimulation of IL-2 production by Ramos.DR4 cells incubated with HSA in the presence of the 17 kDa extract (Figure 6(b)). These results suggest that bryostatin-1 treatment upregulates expression of a 17 kDa protein in BL, and this protein has an immunostimulatory function.

4. Discussion

BL possesses a well-known defect in HLA class I-mediated Ag presentation, resulting from the poor immunogenicity of the EBNA1 protein. EBNA1 possesses an internal Gly-Ala repeat that impairs its proteasomal processing, leading to weak stimulation of CD8+ T cells [35]. This defect, although well studied, addresses only one aspect of the immune response to BL. Less is known about the role of HLA class II-mediated immune responses to this malignancy. Studies have generally focused on CD8+ T-cell responses due to their ability to



(a) HSA_{64-76k} peptide presentation

(b) HLA-DR4-HSA_{64-76K} peptide binding

Figure 5: Bryostatin treatment increased class II peptide binding and restored class II Ag presentation in BL cell lines. (a) The BL cell lines Nalm-6.DR4 and Ramos.DR4 were treated with 0, 20, or 40 nM bryostatin-1 for 24 h. Following treatment, cells were collected, washed, and incubated with HSA_{64-76K} synthetic peptide for 24 h. Cells were then washed and co-cultured with the T-cell hybridoma 17.9. Supernatant from the co-culture was assayed by ELISA to determine IL-2 levels as a measure of T-cell stimulation. Results from these assays show that bryostatin-1 treatment significantly restores Ag presentation in both Nalm-6.DR4 and Ramos.DR4 to levels comparable to B-LCL. (b) Ramos.DR4 cells were treated with 40 nM bryostatin overnight and then washed, fixed, and incubated with 0 μM, 10 μM, or 40 μM of b-HSA for 3 h at 37°C with shaking. The cells were then collected and lysed, and class II/peptide complexes were detected by ELISA. $^*P < 0.001$.

directly kill target cells, but CD4+ T-cell responses mediated by class II are needed for lasting immune responses and memory [36, 37].

In this study, we have shown that although BLs express measurable class II proteins on their cell surface, they were unable to stimulate CD4+ T cells through presentation of HSA peptide or epitope. We demonstrate further that when incubated in buffer at pH 5.5, BL cells regain class II-mediated Ag presentation capacity. Treatment of BL with bryostatin-1 led to restoration of class II presentation and CD4+ T-cell stimulation. This restoration was due, in part, to the upregulation of a 17 kDa, immunostimulatory, peptidylprolyl-like protein which is normally expressed at very low levels in BL, but highly expressed in B-LCL.

The efficiency of class II-mediated Ag presentation to CD4+ T cells may be partially affected by the expression levels of components in the class II pathway: Ii (invariant chain), HLA-DM and HLA-DO [26, 33, 38]. However, we did not observe any significant differences in the expression levels of these pathway components between two BL and two B-LCL cell lines, ruling this out as contributing to the observed defect in BL. CD4+ T cell activation is also dependent on signals delivered by the co-stimulatory molecules, CD80/86, expressed by B cells, yet BLs are known to express lower levels of these molecules [39]. It is plausible that the BL-associated class II defect was a result of insufficient co-stimulation. External co-stimulation may be provided to T cells in the form of anti-CD28, which serves as a surrogate for CD80/86. While our T-cell hybridomas do not require co-stimulation, we still evaluated whether the decreased expression of co-stimulatory molecules contributed to the BL defect. However, even under these conditions, BL cells were unable to stimulate activation of CD4+ T cells. We gleaned further evidence that co-stimulation is not the cause the BL-associated class II defect from assays demonstrating that cross-linking IgM on BL cells failed to result in CD4+ T-cell stimulation (data not shown).

The presentation of Ag via HLA is central to the immune system's ability to detect pathogens and transformed cells and mount immune responses to these cells. Efficient Ag presentation is dependent on efficient binding of peptides to HLA. While co-stimulation was not the cause of BL's inability to present Ag through class II, the possibility existed that BL-expressed class II was not able to bind Ag efficiently, thus preventing T-cell stimulation. Binding assays, however, demonstrated that BL and B-LCL both bound peptide with a similar, measurable affinity.

Bryostatin-1, a potent modulator of protein kinase C, has previously been shown to stimulate upregulation of HLA class II in colorectal cell lines and dendritic cells and to enhance Ag presentation in dendritic cells [23, 24]. To date, however, its effect on HLA class II expression

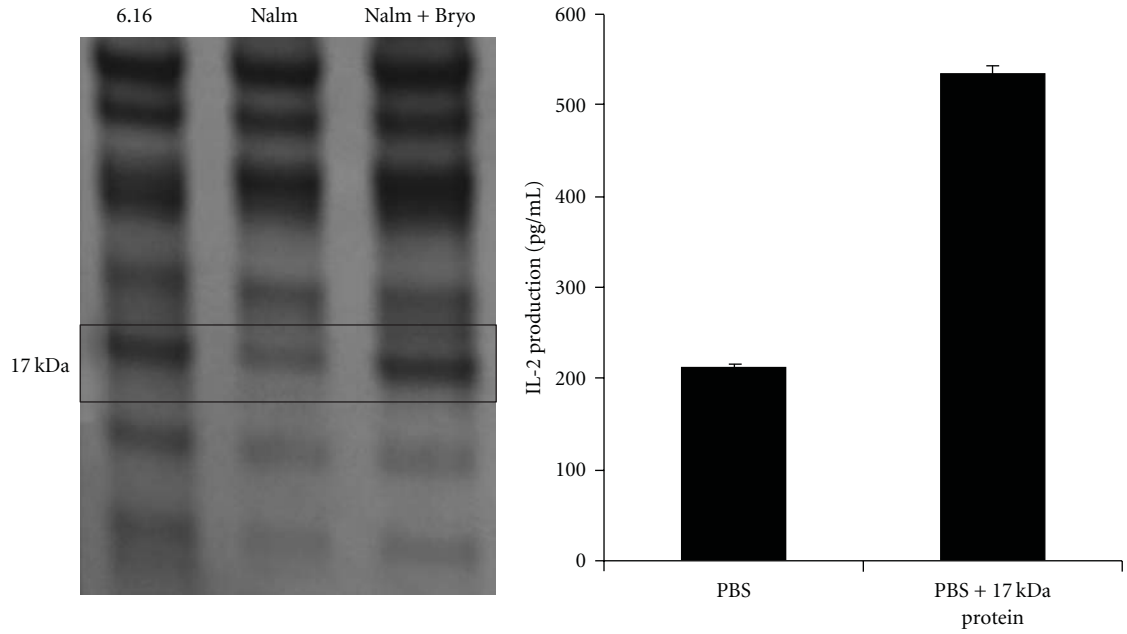


Figure 6: Treatment of BL with bryostatin-1 causes upregulation of a 17 kDa immunostimulatory protein. (a) Acid eluate from Nalm-6.DR4, Nalm-6.DR4 treated for 24 h with 40 nM bryostatin-1 and 6.16.DR4.DM were collected, and subjected to SDS-PAGE, followed by coomassie blue staining. Gel banding patterns revealed upregulation of a 17 kDa protein in bryostatin-treated Nalm-6.DR4 that is expressed at low levels in untreated Nalm-6.DR4 but highly expressed in 6.16.DR4.DM. (b) Acid eluate from 6.16.DR4.DM was separated on a large, non-reducing gel, the band corresponding to 17 kDa was excised, and the protein was extracted by sonication in PBS on ice. Ramos.DR4 cells were then incubated in PBS or 17 kDa gel extract with $HSA_{64–76K}$ synthetic peptide. Cells were then washed and co-cultured with the T-cell hybridoma line 17.9. Supernatant from the co-culture was assayed by ELISA to determine IL-2 levels as a measure of T-cell stimulation. Results show that IL-2 stimulation is significantly increased in Ramos.DR4 cells incubated with peptide in the presence of the 17 kDa gel extract. *indicates $P < 0.05$.

and Ag presentation in lymphoid malignancies has not been evaluated. Based on this information, we sought to determine if treatment with bryostatin-1 was sufficient to enable restoration of class II-mediated Ag presentation to BL. We found that the BL cell lines Nalm-6.DR4 and Ramos.DR4 did indeed regain HLA class II Ag presentation capacity following treatment with bryostatin-1. Our assays with Ramos.DR4 cells demonstrated that restoration of HLA class II Ag presentation could partially be due to increased peptide binding by HLA class II following treatment with bryostatin-1. Additionally, protein expression analysis following bryostatin-1 treatment showed a marked increase in a 17 kDa peptidylprolyl-like protein in Nalm-6.DR4, which was expressed at very low levels in untreated Nalm-6.DR4 and expressed at high levels in 6.16.DR4.DM. This protein, when extracted and used in Ag presentation assays, enhanced class II-mediated Ag presentation in 6.16.DR4.DM cells. Thus, bryostatin-induced restoration of class II Ag presentation in BL cells was mediated by an increased expression of a peptidylprolyl-like protein.

BL is a rapidly growing malignancy and thus requires aggressive chemotherapy to control its spread. Currently used chemotherapy regimens have achieved high cure rates in both adults and children, but treatment-associated toxicities are problematic. This issue is of particular concern for elderly and HIV-infected patients who show inferior responses and reduced tolerance of treatment-associated toxicities [40]. Treatment success has improved with the use of anti-CD20 monoclonal antibody, rituximab [41]. However, toxicities remain problematic, and the use of an immunosuppressive in HIV-infected patients is a subject of ongoing debate [42, 43]. While current treatments for BL have shown overall success, there is obvious room for improvement in the treatment of elderly and HIV-infected patients. Our future studies will continue to evaluate the role of bryostatin-1 in restoring class II-mediated Ag presentation in BL and determine the immunostimulatory role of the peptidylprolyl-like protein. A better understanding of these factors may lead to development of novel immunotherapies which could augment, lessen, or eliminate the need for toxic chemotherapies.

Authors' Contribution

Azim Hossain and Jason M. God contributed equally to this paper.

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Review Article

Immune Microenvironment in Colorectal Cancer: A New Hallmark to Change Old Paradigms

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Impact of immune microenvironment in prognosis of solid tumors has been extensively studied in the last few years. Specifically in colorectal carcinoma, increased knowledge of the immune events around these tumors and their relation with clinical outcomes have led to consider immune microenvironment as one of the most important prognostic factors in this disease. In this review we will summarize and update the current knowledge with respect to this intriguing and complex new hallmark of cancer, paying special attention to infiltration by T-infiltrating lymphocytes and their subtypes in colorectal cancer, as well as its eventual clinical translation in terms of long-term prognosis. Finally, we suggest some possible investigational approaches based on combinatorial strategies to trigger and boost immune reaction against tumor cells.

1. Introduction

The term immunity derives from the Latin word "*immunitas*", referred to the exemption of Roman senators in legal procedures while holding their public office. In time, this term has won many other meanings; in the Medical field it is employed to describe the reaction of an organism towards the aggression caused by external pathogens, initially infectious agents. More recently, antigens derived from neoplastic processes have been reported as responsible for triggering immune responses. Most solid tumors induce an immune response in the host, confirmed by histopathological studies. In this sense, tissue affected by colorectal cancer is invaded by immune cells from the host, suggesting that the amount of lymphocytes may play a prognostic role with a potential impact upon patient's survival [1].

In Europe, 376.000 new cases of colorectal cancer are diagnosed each year, with mortality close to 203.700 patients. It is one of the most frequent cancers worldwide, in both genders [2, 3], and in most developed countries; as a result of screening and diagnostic techniques and advances in the field of surgery and radio-chemotherapy, survival has significantly increased in the last decades. Most tumors affecting the colorectal area are adenocarcinoma-like which in most cases are well or moderately differentiated. If colorectal neoplasias invade through the muscularis mucosa into the submucosa, local host reactions take place in cancer tissue and proinflammatory cells accumulate along the margins of the tumor, creating an immune microenvironment and triggering an immune response targeted towards the tumor [4].

2. Tumor Immune Microenvironment: Immune-Surveillance and Tumor-Infiltrating Lymphocytes (TILs)

In normal conditions, the immune system is an effective "gate-keeper" against cancer. Antitumor activity of the immune system is initially mediated by innate immunity,

mainly with effector cells such as Natural Killer (NK) cells, neutrophils, and macrophages. Subsequently, adaptive immunity mechanisms are activated. This response is specific and generates memory cells, mainly B and T-cells which encompass the humoral and cellular immunity [1].

Cancer development can be explained, at least in part, by the success of the immunosuppressive escape mechanisms displayed by the tumor against the host's immune response [5]. This scenario is an area of great interest in the research of tumor microenvironment, with evidence supporting the hypothesis that a potent and effective immune reaction against certain tumor antigens (epitopes) may overcome escape mechanisms, leading to the elimination and control of the cancer [5]. The aforementioned evidence led to Hanahan and Weinberg, among others, to postulate recently avoidance of immune-surveillance as a new hallmark of cancer [6]. In this sense, cancer cells may escape the innate and immune host responses mainly by two mechanisms: selection of non-immunogenic tumor cell variants (immunoselection) or by active suppression of the immune response (immunosubversion) [5, 6].

Tumor-infiltrating lymphocytes (TILs) are located in the inflammatory infiltrates in tumor islets and in the peritumoral stroma of solid tumors [7]. TILs include cytotoxic T-lymphocytes (CD8), NK cells, and helper T-lymphocytes (CD4). Among the latter, there is a subpopulation of cells known as regulatory T-cells (Tregs), formerly suppressor T-cells, main actors in suppressing and controlling the immune response [8]. Whereas Treg cells carry out a physiological role in the prevention of autoimmune events in the host to avoid a disproportionate response to self antigens, in the case of malignant neoplasias their presence seems more related to immunosuppressive mechanisms preventing immunomediated tumor destruction [9]. The relationship between CD8/NK and Treg cells in the tumor-peritumor microenvironment offers an explanation to the final effect of a triggered immune response with an effective response or an immunosuppressive effect resulting in tolerance-anergy [10].

3. The Immune Synapses: Role of the Antigen 4 Associated to Cytotoxic T Lymphocytes (CTLA-4)

The immune system is a homeostatic system with self-regulating mechanisms that prevent excessive and harmful responses towards the organism that lead to the destruction of normal and healthy cells [1]. One of the key control points in this immune response relies in the HLA-antigen complex recognition by T-cell receptors. This interaction is very complex and involves a series of ligands, such as CD40, a surface molecule that appears early in activated T-cells [7]. This ligand is essential in the generation of antibodies by T-cell induced B lymphocytes as well as in the activation of antigen presenting cells (APCs) which trigger cellular immune responses. The interaction between CD40 ligand and receptor on B-cells and APC upregulates the expression of two surface proteins, CD80 and CD86. When these interact with CD28 on T-cells (immune synapses), T-cells are activated [7]. However, interactions with antigen 4 associated to cytotoxic T lymphocytes (CTLA-4) on T-cells lead to a status of anergy or immune tolerance. Once CTLA-4 (CD152) is generated, immune synapses are mobilized 2-3 days after T-cells are activated, binding to T-cell receptors (TCRs) CD80 and CD86 [11] (Figure 1). CD80 and CD86 preferentially bind to CTLA-4, leading to a decrease in IL-2 production, thus, in activated T-cells. A temporary delay in CLTA-4 appearance on T-cell surface in the immune synapses may trigger RCT and CD28-induced LT activation and expansion, enhancing the immune response [7, 12].

The blockade of CTLA-4 interaction with its ligands can result in an augmentation of antigen specific T-cell responses [13], and several studies have demonstrated that CTLA-4 blockade can enhance immunity to tumors [14, 15]. It has been reported that antibodies against CTLA-4 (anti-CTLA-4) induce proliferation of TCR-stimulated T effector cells and abrogate Treg suppressive activity by enhancing IL-2 and IFNγ release in response to polyclonal or tumor antigen stimulation [16]. Curiously, anti-CTLA-4 does not reduce the amount of Tregs, what suggests that anti-CTLA-4 mediates immune responses by direct activation of T effector cells and not by depleting Tregs [16].

There exist 2 CTLA-4 blocking antibodies for use in humans that have been most widely tested in patients with metastatic melanoma [17]. Recently, Ipilimumab has gained FDA approval for clinical use in metastatic melanoma patients after demonstrating benefits in overall survival [18]. Clinical research of anti-CTLA-4 in other solid neoplasms is scarse until now. However, a better understanding of the mechanism of action of anti-CTLA-4, along with its use in the context of combinatorial strategies, may enable to explore the eventual efficacy of these molecules in nonmelanoma tumors, including colorectal cancer [19].

4. Prognostic Value of Tumor-Infiltrating Lymphocytes (TILs) and Their Subtypes in Colorectal Cancer

Microscopically, lymphocytes are observed as small cells responding to classical hematoxylin-eosin stains and clearly different from other white cells such as plasmatic cells, neutrophils, eosinophils, macrophages, and masT-cells. In a study published in 1987 by Jass et al. [20], they reported the possibility that lymphocytes infiltrate of the invasive margins of rectal cancer could be an independent prognostic factor for survival, advocating for a new prognostic tool to calculate the risk of this disease. Ropponen et al. [21] confirmed the prognostic value of TILs in colorectal cancer, quantifying them in the tumor stroma and along the invasive margins of the tumor. They subdivided them into four groups according to their histological grade and proved that TILs infiltration was a predictive factor for disease-free and overall survival. An inverse correlation was also observed between the presence of TIL and tumor stage; thus in advanced stages of the disease (Dukes stages C and D),

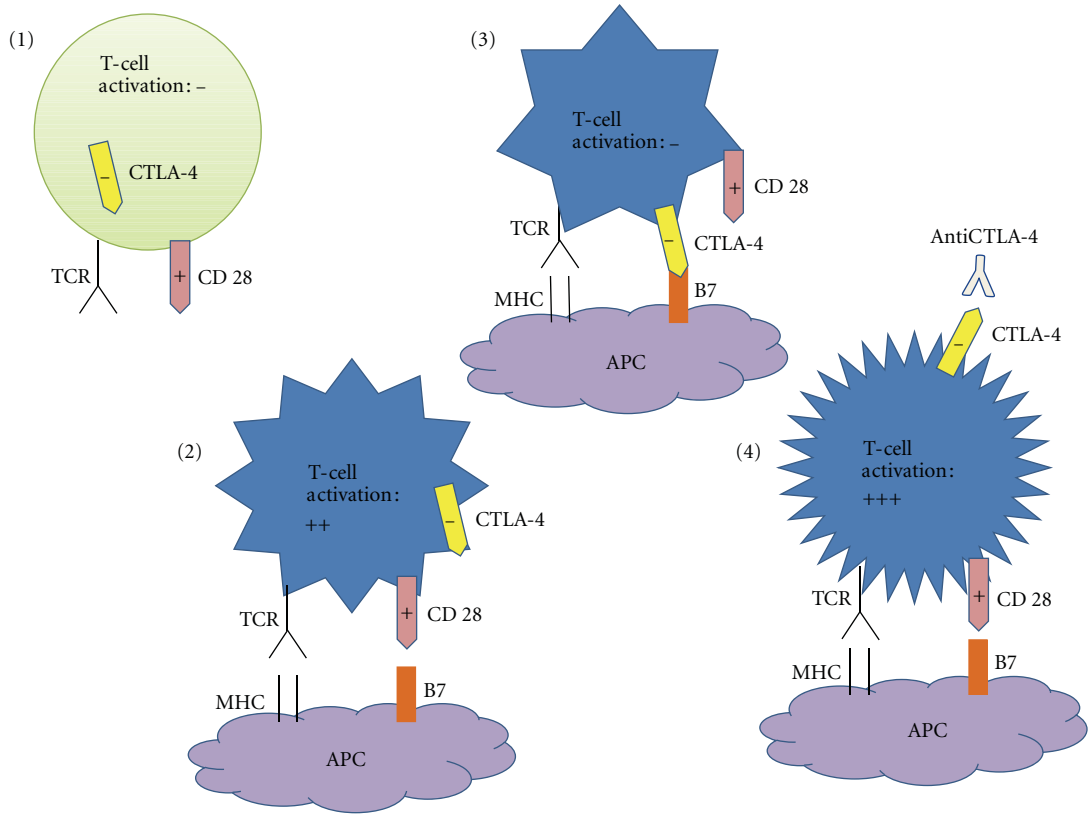

Figure 1: (1) CTLA-4 is a negative regulator of T-cell activation. (2) Conventional T-cells are activated by engagement of MHC and B7. (3) Upon activation, T-cells express CTLA-4 on the cells surface and the union of CTLA-4 with B7 inhibits T-cell activation. (4) Antibody blockade of CTLA-4 produces the liberation of CD28 which could engage with B7 with the best activation of T-cells.

TILs were less numerous than in early stages (Dukes stages A and B) [21].

Follicular and paracortical hyperplasia in local lymphatic nodes are also an important prognostic factor in colorectal cancer. Phil et al. [22] proved in their study that immune response observed in local lymphatic tissue might exert an influence on survival. This study is particularly important as it establishes a correlation between the immune response observed in the tumor layer and in the first lymphatic settlement. Both immune responses are directly related; hence immature dendritic cells migrate from the primary tumor location to the local lymphatic node for maturation and conversion to T-cell antigen presenting cells [22].

In most colorectal tumors, tumoral tissue is infiltrated by a scarce number of lymphocytes and only along the margins of the tumor the highest density of lymphocytes and other inflammatory cells is observed. Proinflammatory cells such as neutrophils and macrophages usually appear with lymphocytes. The latter are usually CD4+ or CD8+ T-cells while B-cells are generally observed in lymphoid follicles [1].

The specific TILs composition has a crucial role in clinical evolution of colorectal cancer. Many research groups have focused their effort on analyzing the eventual relation between T effector cells and regulatory T-cells infiltrates and clinical outcomes. Intraepithelial lymphocytes are mainly CD8 and their number is consistently correlated with higher disease-free survival rates, as proved in several studies [4, 23]. On the contrary, studies that analyze Tregs infiltration report conflicting results [24].

4.1. Regulatory T-cells. Treg population represents roughly the 10% of CD4 T-cells and specifically expresses the forkhead box P3 transcription factor (FOXP3) [25, 26] which confers them suppressive properties upon effector T-cells [27, 28]. Increased numbers of FOXP3-infiltrating tumor cell nests have been demonstrated in several neoplasms, and this event is generally associated with unfavourable clinical outcomes. However, there are tumors where Treg infiltration seems to play a different role with protective antineoplastic effects. This is the case of some lympho-proliferative syndromes, especially Hodgkin's disease and follicular lymphoma [29], and probably (but less clear) in colorectal and head and neck carcinomas [30]. Regarding colorectal cancer, Salama et al. [31] after analyzing 967 surgical specimens detected that a high density of Tregs in tumor tissue was associated with better survival, being the only immune biomarker independently associated with overall survival in the multivariate analysis. In the same way, Correale et al. [32] reported a better outcome in advanced colorectal cancer treated with chemo or chemo-immunotherapy if previously there was an intense Tregs infiltration in primary tumors. Two other recent and large

studies reported similar results, with favourable prognosis in populations with high FOXP3 T-cell infiltration, at least in the univariate analysis [33, 34].

Ladoire et al. revised in depth this issue and pretended to give a plausible biological explanation based on the different effects of Tregs populations, depending on the diverse and specific microenvironment composition of the tumors [30]. In this sense, they underscore that colorectal carcinomas grow in a "septic microenvironment" where many gastrointestinal bacteria reside and can be translocated across the mucosal surface, inducing proinflammatory and proangiogenic effects, that favour the tumoral growth. In this context, Tregs may suppress the immune reaction induced by these microorganisms and thus counteract their protumorigenic effects. This is an interesting and attractive hypothesis which may explain the improved outcomes associated with Treg infiltration in some neoplastic diseases (hematologic and solid tumors) that have a tight relation with infectious processes.

Although most of the studies advocate for the beneficial effects of Treg infiltration in colorectal cancer (Table 1), there exist other works that could not fully confirmed these results. Sinicrope [35] reported no significant relation between Tregs and prognosis and observed that a low epithelial CD3+/Tregs ratio was associated with shorter disease-free survival. In addition, Camus et al. [36] did not find Tregs infiltration as a reliable marker of good prognosis. Therefore, to date there exist some conflicting results regarding clinical results and accumulation of FOXP3 Tregs in specimens of colorectal cancer and more data are needed to definitely elucidate and establish their role in this disease.

4.2. Cytotoxic T CD8+ Cells. In relation to Tregs, results regarding CD8+ infiltration in colorectal cancer are more robust and concordant suggesting strong antitumoral effects and a positive effect on patient survival [24] (Table 2). Diederichsen et al. [37] showed throughout flow cytometry that a low CD4/CD8 ratio is an independent prognostic factor for a better survival. The immunosuppressive role of CD4+, CD25+, and FOXP3+ regulatory T-cells is also elucidated [37].

In 2006, Galon et al. published in Science [39] a very relevant study with clinical-pathological transcendence. Genomic analyses were conducted on 75 cases of colorectal carcinoma in stages I to III and 415 cases with tissue microarrays, observing that tumors with lower rates of recurrence had higher density of immune cells (TCD3, TCD8, memory-TCD45RO, and granzyme B) in the analyzed regions in comparison to recurrent tumors. This study shows that adaptive immunity, expressed by Th1, is inversely proportional to tumor recurrence; thus patients with increased Th1 gene expression present a better prognosis. Furthermore, the centre and margins of the tumor were analyzed finding that, in patients without recurrence, immune cell density was higher in both areas. In patients with low density of total lymphocytes TCD3 and memory lymphocytes (CD45RO+) presented a worse prognosis, similar to those with distant metastasis (stage IV). Patients were stratified according to the UICC-TNM classification, observing that an intense immune response in situ was related to a favourable prognosis despite local extension of the tumor and nodal locoregional infiltration [39]. The authors finally advocate for a redefinition of the diagnostic and histopathological approaches of these tumors as long as immune cell type, density, and location in colorectal carcinoma proved to be a superior prognostic factor and independent from classical prognostic factors in this neoplasia (stage according to the UICC-TNM classification and nodal infiltration). However it is important to notice that it is not possible to absolutely discard an unbalanced selection of the cases due to a higher number of tumors carrying DNA microsatellite instability (MSI) in this study.

Multiple analyses clearly point out that the impact on survival of CD8+ lymphocytes in colon cancer is more obvious with longer follow-up periods [42]. Moreover, in follow-up studies conducted on patients with high or low levels of CD8, survival curves during the first two years are very similar, further separating [24]. Chiba et al. [38] proposed the hypothesis that the presence of CD8+ T-cells in tumor tissue could trigger an immunosurveillance status in the organism, avoiding the development of distant metastasis. Pagès et al. [40] proved that early metastasis development was associated with a poor immune response in tumor tissue. This group demonstrated in 490 patients of colorectal cancer that those patients with a high density of CD45RO+ cells had better prognosis in terms of disease free and overall survival compared with patients with a low density of these memory cells. Tumors without signs of early metastatic invasion had increased infiltrates of immune cells, particularly CD8+ T-cells [40]. Furthermore, Pagès et al. [43] reported in 2009 another study in which they classified 602 early-stage colorectal cancers (stage I and II) into different prognostic groups depending on the density of CD45RO+ and CD8+ cells in two tumor regions (center and invasive margin). Immune classification was found to be an independent prognostic factor in multivariate analysis ($P < 0.0001$), revealing recurrence rates of 4,8% versus 75% in high versus low CD8+ and CD45RO+ infiltration, respectively [43]. Similarly, Mlecnik et al. [44] studied the intratumoral immune infiltrates in a broader population of stage I to IV colorectal cancers, measuring again the lymphocyte infiltrates in the center and the invasive margin of 599 specimens. They used the same immune score of their previous study, defining five patient groups (Im0, Im1, Im2, Im3, Im4). Patients with low densities of CD45RO and CD8 in both tumor regions were classified Im0, and the rest of groups were classified depending upon the density in every tumor region up to the group of four high densities (Im4). In this population, disease free survival and overall survival was far better in the Im3 and Im4 groups, and multivariate analysis confirmed the advantage of the immune score (HR 0,64; $P < 0,001$) compared with the classical TNM staging [44].

4.3. DNA Microsatellite Instability. Another issue worthy of consideration is the well-recognized better prognosis of patients with colorectal cancer in the context of Lynch's syndrome [41]. In this sense, DNA microsatellite instability

Table 1: Studies of tumor-infiltrating immune cells (Tregs) and prognosis in colorectal carcinoma.

Study	*n*	Immune cells	Findings: correlation with prognosis
Salama et al. [31]	967	CD8+, CD45RO+, and FOXP3+ tumor-infiltrating	Positive correlation for T-regs in tumor, negative in normal mucosa
Correale et al. [32]	57	CD4+, CD8+, and FOXP3+ T-cells in stroma adjacent to neoplastic glands	Positive correlation for T-regs
Sinicrope et al. [35]	160	CD4+, CD8+, CD25+, and FOXP3+ T-cells	Negative correlation for T-regs. Positive correlation for CD3+ T-cells
Frey et al. [33]	1420	FOXP3+ T-cells	Positive correlation for FOXP3+ T-cells
Nosho et al. [34]	768	CD3+, CD8+, CD45RO+, and FOXP3+ T-cells	Positive correlation for CD8+, CD45RO+, and FOXP3+ T-cells.

Table 2: Studies of tumor-infiltrating immune cells (Cytotoxic T CD8+ cells and CD45RO+) and prognosis in colorectal carcinoma.

Study	*n*	Immune cells	Findings: correlation with prognosis
Naito et al. [4]	131	CD8+ and GrB+ tumor-infiltrating cells.	Positive correlation for CD8+ T-cells
Jass et al. [20]	104	Tumor-infiltrating S-100+, HLA class II+, CD208+, CD1a+ dendritic cells.	Negative correlation for dendritic cells
Chiba et al. [38]	371	CD8+ T-cells within cancer cell nests	Positive correlation for CD8+ T-cells
Galon et al. [39]	490	CD3+, CD8+, GrB+, and CD45RO+ lymphoid infiltrates in tumors/invasive margin	Positive correlation for CD8+ and CD45RO+ T-cell
Pagès et al. [40]	490	CD3+, CD8+, GrB+, and CD45RO+ lymphoid infiltrates in tumors/invasive margin	Positive correlation for CD45RO+ T-cells
Camus et al. [36]	142	CD3+, CD5+, CD8+, CCR+, CD1a+, Ki67+, CD68+, FOXP3+, and cytoDEATH+ tumor-infiltrating cells	Positive correlation for CD8+ and CD45RO+ T-cells
Guidoboni et al. [41]	109	CD3+, CD8+, and GrB+ tumor-infiltrating cells	Positive correlation for CD8+ T-cells
Menon et al. [23]	93	CD4+, CD8+, CD56+, and CD57+ intraepithelial cells.	Positive correlation for CD8+ and CD57+ cells
Diederichsen et al. [37]	41	CD3+, CD8+, and CD4+ tumor-infiltrating cells	Positive for CD8+ T-cells, negative for CD4+ T-cells
Ogino et al. [42]	843	Lymphocytes on top of tumor cells	Positive correlation for lymphocytes
Ropponen et al. [21]	276	Lymphocytic infiltration in the center and periphery of tumors	Positive correlation for lymphocytes

is frequently observed in these hereditary nonpolyposic colorectal cancers and by contrast is relatively uncommon in sporadic colorectal tumors. Usually, tumor epithelium in cases with microsatellite instability is infiltrated by CD3+ and CD8+ lymphocytes, probably resulting from an increased immunologic reconnaissance of mutated proteins on the epithelial surface [45]. Several studies have revealed that microsatellite instability can be associated with a greater T-cell infiltration in tumor tissue [41, 45–47], and hence there has been postulated the hypothesis that this fact might be on the basis of the better clinical outcomes associated with this subgroup of hereditary colorectal cancers. Although this is a plausible explanation, other further prospective studies focusing on histopathologic findings in patients with hereditary nonpoliposic colorectal carcinomas might clarify this question.

4.4. Antigen Presenting Cells (APCs). Along with TILs, antigen presenting cells (APCs) are another components of adaptive immune system worthy of consideration, and among them dendritic cells (DC) are retained as the most potent antigen presenting cells. At present there are numerous studies investigating their role in order to use them in active immunotherapy (vaccines). In colorectal cancer, dendritic cells are found along the invasive margins of the tumor once they have developed completely in lymphoid follicles [48]. The prognostic value of these cells is very important. Dadabayev et al. [49] published that HLA-II cells are distributed in the tumor stroma and that in cases with high density of HLA-II cells, survival was lower; this may be due to the fact that HLA-II cells in those cases are immature as mature cells are scarce in tumor regions. Moreover, overexpressed intercellular adhesion molecule ICAM-1 in tumor

stroma fibroblasts could interfere in dendritic cell functions [50]. It is important to remind that tumor reactive T-cells are often anergic because of inappropriate antigen exposure or owed to self recognition; so DCs concourse seems essential to trigger immune-mediated antitumor responses with the ability to generate effector and memory T-cells.

5. Immune Effects of Chemotherapy in Colorectal Carcinoma

Colorectal cancer represents a wide group of heterogenic diseases with different clinical behaviours and response to antineoplastic treatments. Nowadays, the main option in advanced disease remains chemotherapy or biochemotherapy. Recently, several studies have revealed that these treatments seem to have a relevant impact on the surrounding stroma and microenvironment [51]. Different cytotoxic drugs destroy tumor cells inducing a type of immunogenic apoptosis, a process of cell death characterized by the activation of caspases and exposure of phosphatidilserine residues in the outer leaflet of the cell [52], and recent studies suggest that this kind of tumoral destruction may improve cancer cell recognition by the immune system [53, 54].

Apoptosis or programmed cell death has been traditionally considered as immunologically "bland" or non-immunogenic. However, this theoretical assumption has not been confirmed in basic and translational research. Rather, it seems that apoptosis is a heterogeneous process that under some circumstances may lead to immunogenic effects [55–57], and this finding is critical to understand better the antineoplastic mechanism of action of some, if not most, chemotherapies.

Oxaliplatin is one of the drugs of choice in advanced colorectal cancer and is included in most of the first line chemotherapy schedules. The group of L. Zitvogel at the Institut Gustave Roussy have studied extensively the immunogenic death of cancer cells induced by chemotherapy, and with respect to oxaliplatin they have demonstrated that it may promote apoptosis in cancer cells via immunogenic effects through two main mechanisms [58, 59].

(1) *Early Apoptotic Phase: Calreticulin* (*CRT*). Oxaliplatin induces translocation of the intracytoplasmic protein calreticulin to the cell surface, inducing the apoptotic cell antigen presentation to dendritic cells and stimulating specific antitumor T-cell responses [58, 59].

(2) *Late Apoptotic Phase: High Mobility Group Box 1* (*HMGB1*). Another immunogenic determinant of cell death is the proinflammatory factor HMGB1. HMGB1 is a nuclear protein that is released after necrotic cell death and, as recently reported, from dying cells during late stage apoptosis. After death cell induced by oxaliplatin, HMGB1 may be released in the stroma and act as a neoantigen representing an immunogenic endogenous "danger signal", and thus initiating an inflammatory response through binding Toll-Like Receptor 4 (TLR4) on DC [59].

Therefore, immunogenic tumor cell death mediated by chemotherapeutics like oxaliplatin is a multistep process characterized by a temporal sequence of events (Figure 2) including early translocation of calreticulin to the cell surface, and thereafter interaction of CRT with multiple receptors on DC with apoptotic bodies phagocytosis, release and exposure of heat shock proteins, and late release of HMGB1 (60). HMGB1 is able to bind to the TLR4 receptor on DC, which allows tumor-derived antigens to be processed and presented along with MHC and costimulatory molecules on the surface of DC [53, 60]. These mechanisms altogether serve to trigger DC-mediated specific antitumor response, which may be enhanced by the use of costimulatory molecules like GM-CSF or interleukins [7, 61].

Therefore, in contrast with the previous theoretical assumptions, chemotherapeutics like oxaliplatin can induce a highly potent immune response by increasing neoantigen threshold and presentation via antigen presenting cells, with enhancement of T-cell response and generation of memory T-cells [55, 57]. This new paradigm may serve to consider chemotherapeutics as less empirical and more specific drugs, and thus it is tempting to speculate that systemic treatments in colorectal cancer might be customized taking into account their potential effects on tumoral microenvironment. In this sense, there is an interesting field of clinical research to discover that may combine classical CT agents with immunogenic effects with boosting cytokines (GM-CSF, IL2) and new immunogenic molecules like monoclonal antibodies anti-CTLA4 and CD40 agonists. These combinatorial strategies may eventually sustain immunogenic effect of tumoral cell death, enhancing antigen recognition and thus increasing the effector and memory cells specific activity. Regarding this, biomarkers of immune activity should be of the greatest interest, in order to serve as proof of principle of efficacy with an earlier detection of the eventual benefits of oncological treatments in patients. In this sense, changes detected during CT treatments in blood samples, especially in immunophenotype, Tregs amount, and TCD8/Tregs ratio, may represent interesting biomarkers to analyze and validate in the future.

6. Conclusions

Scientific evidence supporting the importance of the immune response in neoplastic diseases is growing. In colorectal carcinoma, many studies endorse the prognostic value of TILs infiltration density, depending on the specific subtype of lymphocytes present. Thus, higher densities of effector TCD8 and NK cells in tumor islets and peritumoral tissue seem to be associated with better long-term survival rates.

Despite active research in this field is ongoing and there remain many issues still unsolved, available data support the realization of a systematic histopathological study of the tumor microenvironment along with the classical pathological studies in colorectal cancer. In addition, immune microenvironment may represent a new oncological target from a therapeutic perspective, giving rise to a new promising chance of clinical research to our patients.

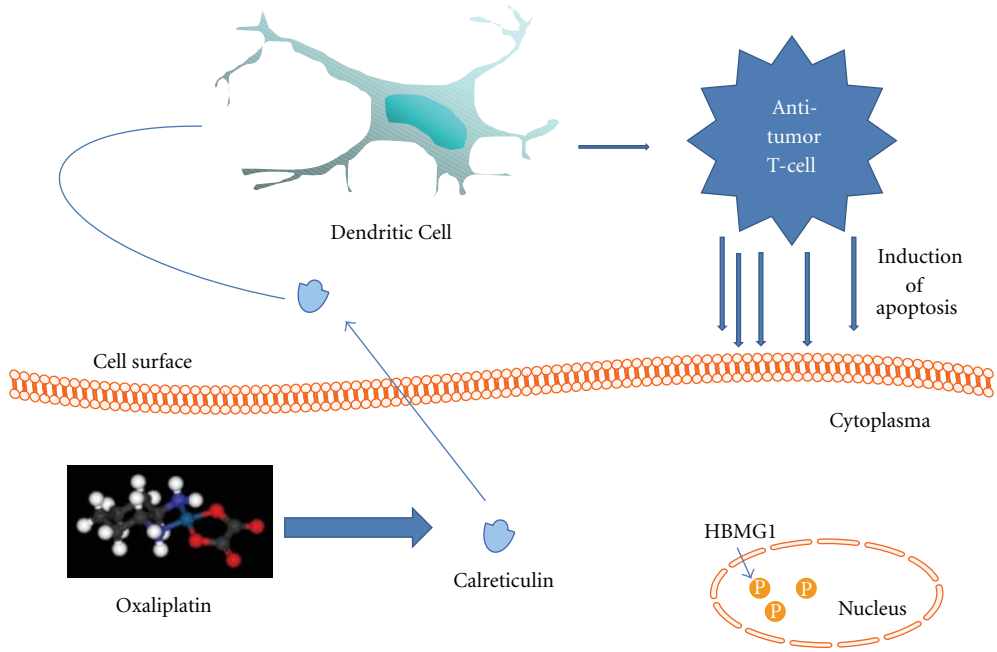

Figure 2: Early Apoptotic Phase: Calreticulin (CRT). oxaliplatin induces translocation of the intracytoplasmic protein calreticulin to the cell surface, inducing the apoptotic cell antigen presentation to dendritic cells and stimulating specific antitumor T-cell responses.

Conflict of Interests

The authors declare that there are no conflict of interests.

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Review Article

Tumor Cells and Tumor-Associated Macrophages: Secreted Proteins as Potential Targets for Therapy

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Inflammatory pathways, meant to defend the organism against infection and injury, as a byproduct, can promote an environment which favors tumor growth and metastasis. Tumor-associated macrophages (TAMs), which constitute a significant part of the tumor-infiltrating immune cells, have been linked to the growth, angiogenesis, and metastasis of a variety of cancers, most likely through polarization of TAMs to the M2 (alternative) phenotype. The interaction between tumor cells and macrophages provides opportunities for therapy. This paper will discuss secreted proteins as targets for intervention.

1. Introduction

Inflammatory pathways, meant to defend the organism against infection and injury, as a by-product, can promote an environment which favors tumor growth and metastasis. Several infections, inducing inflammation, have been directly linked to cancer. Well-known examples are *Helicobacter pylori* infection and gastric cancer [1], hepatitis B and C virus and hepatocellular carcinoma [2], and schistosomiasis and bladder cancer [3]. Inflammation has therefore been coined the seventh hallmark of cancer [4–7].

Macrophages are among the first cells to infiltrate infected or damaged tissue [8]. Tumor-associated macrophages (TAMs), which constitute a significant part of the tumor-infiltrating immune cells, have been linked to the growth, angiogenesis, and metastasis of a variety of cancers, most likely through polarization of TAM to the M2 (alternative) phenotype. M1 (classical) macrophages are generally characterized by interleukin IL-12^{high}, IL-23^{high}, and IL-10^{low} phenotype. They produce reactive oxygen and nitrogen intermediates as well as inflammatory cytokines and play a role in Th1 responses. Finally, M1 macrophages mediate resistance against intracellular parasites and tumors. M2 macrophages (characterized by an IL–12^{low}, IL-23^{low}, IL-10^{high} phenotype) are diverse, but in general are involved in T helper 2 (Th2) response, have an immunoregulatory function, and orchestrate encapsulation and containment of parasites and promote tissue repair, remodeling, and tumor progression. Further subdivision of M2 macrophages into M2a (after exposure to IL-4 or IL-13), M2b (immune complexes in combination with IL-1beta or LPS), and M2c (IL-10, TGFbeta or glucocorticoids) has been suggested [9].

Whereas the vast majority of studies with numerous tumor types, including follicular lymphoma [10], intestinal type gastric cancer [11], pancreatic cancer [12], non-gynecologic leiomyosarcoma [13], and thyroid cancer [14], show that the presence of TAM in the tumor microenvironment is associated with a worse prognosis, some studies claim the opposite [15]. The specific role of TAMs in colon cancer is more controversial, as most studies indicate that peritumoral TAMs prevent tumor development (suggesting polarization of TAMs towards the M1 phenotype); patients with high TAM numbers have better prognosis and survival rate [16–19]. In contrast, intratumoral TAM count has been correlated with depth of invasion, lymph node metastasis, and staging of CRC, suggesting that intratumoral macrophages cause cancer cells to have a more aggressive behavior [20, 21].

These contradictions may be due to differences in tumor biology of different tumor types, but may also be a consequence of markers used for the study of TAM. Frequently, the pan-macrophage/monocyte marker CD68 is used as a marker for TAM, whereas the use of CD163 or CD204 might

be more appropriate. In fact, Ohtaki et al. [22] show that whereas presence of CD68+ macrophages was of marginal prognostic significance ($P = 0.08$) in lung adenocarcinoma, the use of CD204 showed a strong association with poor outcome in these patients ($P = 0.007$). Similarly, Espinosa et al. found a very strong association between higher number of CD163+ TAM and myometrial invasion of endometrioid carcinoma. Furthermore, there was a positive correlation between the number of CD163+ TAM in the primary tumor and in regional lymph node metastases [23]. In pancreatic cancer, high numbers of CD163- or CD204-positive macrophages were associated with poor prognosis ($P = 0.0171$); however, this was not the case for the number of CD68-positive macrophages [12].

Finally, regardless of the marker used, it is frequently reported that TAMs are associated with prognosis in univariate analysis, but this association is lost in multivariate analysis [24–26]. An exception to this is Hodgkin's lymphoma, where an increased number of CD68+ macrophages outperformed the international prognostic score in multivariate analysis for disease-specific survival [27].

Nevertheless, it is clear that TAMs play an important role in tumor growth and metastasis. This implies that the interaction between tumor cells and TAM provides an opportunity for cancer treatment. In this paper, we focus on secreted proteins as targets for intervention.

2. Secreted Proteins

2.1. CSF-1. The macrophage colony-stimulating factor (CSF-1 or M-CSF) promotes the differentiation and survival of macrophages. The receptor for CSF-1 is a tyrosine kinase receptor encoded by c-fms. Both CSF-1 and the receptor are expressed by tumor cells of different origins [28, 29], and elevated levels are associated with poor prognosis [30–33]. In fact, in epithelial ovarian cancer patients, elevated levels of CSF-1 in serum or ascetic fluid were associated with poor outcome [34], whereas elevated levels after treatment were indicators of recurrence of progression [35].

2.2. CCL2. Chemotactic cytokine ligand 2 (CCL2, also known as monocyte chemoattractant protein 1 (MCP1), monocyte chemotactic and activating factor (MCAF), and monocyte secretory protein JE) is produced in a wide range of tumors [36–39]. Expression of CCL2 is correlated with TAM migration to the tumor, with high expression resulting in higher numbers of TAM, as well as a higher growth rate of tumors after *in vivo* transplantation [40]. Beside the effect on monocytes, CCL2 has also been shown to inhibit the generation of tumor-reactive T cells [41]. Furthermore, prognostic analysis revealed that high expression of CCL2 was a significant indicator of early relapse in human breast cancer patients [42], potentially through the expression of angiogenic factors and activation of matrix metalloproteinases [43]. These protumoral effects of CCL2 are in contrast with the findings of Zhang et al. [44], who showed that early recruitment of monocytes, by high-CCL2-producing tumors as opposed to low-CCL2-producing tumors, inhibits tumor growth.

2.3. TNF. Whilst TNF-alpha was first identified as a soluble factor capable of inducing tumor necrosis [45], various mechanisms have been described by which TNF-alpha may promote cancer growth, invasion, and metastasis [46]. Two receptors for TNF have been described, TNF-R1 and TNF-R2. TNF-R1 is expressed on all cell types, whereas TNF-R2 expression is limited to endothelial and immune cells [47]. Mice deficient in TNFR1 or TNFR2 were exposed to chemicals to induce skin tumor formation. Tumor multiplicity was significantly reduced in TNFR1−/− and TNFR2−/− mice compared to wild-type mice, suggesting that both receptors have protumor activity. However, TNFR1−/− mice were markedly more resistant to tumor development than TNFR2−/− mice indicating that TNFR1 is the major mediator of TNF-alpha-induced tumor formation [48]. Constitutive production of TNF from the tumor microenvironment is a characteristic of many malignant tumors, and the presence of TNF is often associated with poor prognosis. TNF has been shown to induce tumor cell invasion through NF-κB- and JNK-mediated upregulation of migration-inhibitory factor in macrophages and through enhanced MMP production in tumor cells [49]. TNF further enhances cell migration and metastasis through NF-κB-dependent induction of chemokines, interleukins, and intercellular adhesion molecule-1 [49]. NF-κB, therefore, seems to play a key role in a TNF-induced signaling pathway. NF-κB can be activated by many stimuli, including proinflammatory cytokines (IL-1, TNF), bacteria, LPS, viruses, and cellular stresses (UV, radiation, chemotherapeutics) [50, 51]. Cellular targets of NF-κB are cytokines, including TNF (positive feedback loop, chemokines, adhesion molecules, inducible effector enzymes and regulators of apoptosis, and cell proliferation [51]. Hence, NF-κB plays a central role in inhibition of apoptosis and tumor promotion and progression, suggesting that the use of NF-κB inhibitors might be useful in cancer therapy. Similarly, TNF inhibitors have been used for the treatment of inflammatory and autoimmune diseases, but also for the treatment of cancer. Several drugs are available, including infliximab, a human-mouse chimeric monoclonal antibody, golimumab and adalimumab, fully human monoclonal antibodies, certolizumab pegol, the PEGylated Fab fragment of a humanized monoclonal antibody, and etanercept, a fusion of the TNF receptor and an antibody constant region (Fc). Infliximab [52] and etanercept [53] especially are under study in clinical trials for the treatment of cancer. However, treatment of rheumatoid arthritis and Crohn's disease with anti-TNF drugs, and especially the monoclonal antibodies, was shown to be associated with an increased risk of reactivation of tuberculosis [54]. Therefore, before treatment with anti-TNF antibodies is initiated, a latent tuberculosis infection should be ruled out. Furthermore, in line with the important role of TNF in host defense and tumor growth control, in patients with rheumatoid arthritis treated with anti-TNF antibody therapy, the pooled odds ratio for malignancy was 3.3 (95% confidence interval, 1.2–9.1) and for serious infection was 2.0 (95% confidence interval, 1.3–3.1) [55].

2.4. IL-6. Secretion of IL-6 can be induced by exposure of macrophages to LPS, and hence, can be seen as a representative product of the proinflammatory M1-type macrophages. On the other hand, IL-6 promotes cancer cell proliferation while also inhibits apoptosis of cancer cells through activation of signal transducer and activator of transcription 3 (Stat3) [56]. Stat3 is activated by phosphorylation on Tyr-705, which leads to dimer formation, nuclear translocation, and regulation of gene expression. Serine phosphorylation of Stat3, induced by IL-6 stimulation, has been shown to be independent of mitogen-activated protein kinase and sensitive to the Ser/Thr kinase inhibitor H7. PKC delta is likely to be the kinase that phosphorylates Stat3 in response to IL-6 [57, 58]. Additionally, IL-6 acts as an angiogenic factor and has been implicated in many of the same processes as TNF. Notably, during the cross-talk between cancer and inflammatory cells, Stat3 and NF-κB seem to be key transcription factors linking a mutual positive feedback loop and promoting cancer progression [56]. A tissue microarray study on 221 ovarian cancer cases showed that the intensity of IL-6 staining correlated with prognosis [59]. These data provide the rationale for the use of anti-IL-6 antibodies and STAT-3 inhibitors. A number of clinical studies using siltuximab (CNTO 328), a chimaeric anti-IL-6 monoclonal antibody, have been reported [60–63]. Furthermore, a high-affinity fully humanized anti-interleukin 6 monoclonal antibody (mAb 1339) is available and has shown *in vitro* and *in vivo* antimultiple myeloma activity, both alone and in combination with conventional and novel agents against multiple myeloma [64]. Similarly, sirukumab (CNTO 136), a human monoclonal antibody against soluble IL-6, has been investigated in healthy subjects, showing that it is safe and has a low immunogenicity [65]. Finally, a range of STAT3 inhibitors have been tested and shown to have strong growth-inhibitory activity against cancer cell lines *in vitro* and potent antitumor effects *in vivo* (as reviewed by [66]). Currently, two clinical trials are ongoing, evaluating blockade of STAT3 in solid tumors (NCT00696176, phase 0, and NCT00955812, phase 1), but no results are currently available.

2.5. CCL5. Chemokine (C-C motif) ligand 5 (CCL5), also known as regulated upon activation, normal T-cell expressed, and Secreted (RANTES), plays an important role in T-cell proliferation and IFN-γ and IL-2 production, which promotes the differentiation and proliferation of Th1 cells important for immune defense against intracellular infection. It was shown that the prostaglandin E2, secreted by mammary gland tumor cells, but not by normal mammary gland epithelial cells, inhibited CCL5 expression in macrophages in response to LPS, but not to TNF-α stimulation [67]. Furthermore, an inverse correlation between tumoral CCL5 expression and number of macrophages in the tumor microenvironment has been reported [68], which suggests an antitumoral, rather than a protumoral, role of CCL5. However, when an antagonist of the CCL-5 receptors, CCR1 and CCR5, was used in a mouse model of breast cancer, a significant reduction in volume and weight of treated animals versus controls was observed. The antagonist also showed activity against established tumors [69].

2.6. CCL18. Chemokine (C-C motif) ligand 18 (CCL18) is a small cytokine belonging to the CC chemokine family. It was identified, more or less simultaneously, from a range of sources, leading to different names: found highly expressed in lung, it was called pulmonary and activation-regulated chemokine [70] (PARC); based on its similarity to CCL3 it was called macrophage inflammatory protein-4 [71] (MIP-4); after being cloned from dendritic cells, it was called dendritic cell-chemokine 1, [72] (DC-CK1); when macrophages were the source for cloning, it was called alternative macrophage activation-associated CC chemokine-1 [73] (AMAC-1).

CCL18 is predominantly produced by monocytes/macrophages and dendritic cells (DCs). In case of macrophages, expression of CCL18 can be induced both by Th1 signals (i.e., LPS) and by Th2 signals (i.e., IL-4, IL-10, and IL-13). Immunohistochemistry has shown that CCL18 is produced by CD163+ macrophages [74–76]. Immature DCs express high levels of CCL18, but there is controversy on the effect of maturation, with some reports claiming upregulation [77–79] and others claiming downregulation of CCL18 expression [73, 80, 81]. CCL18 is likely to participate in homing of lymphocytes and DC to secondary lymphoid organs. In case of serious inflammation, CCL18 could assist in mounting a primary immune response through the attraction of naïve T cells towards fully matured DCs [82, 83]. However, in the absence of costimulatory molecules, this can lead to the induction of tolerance through the generation of regulatory T cells (Tregs [84–86]). Furthermore, it has recently been shown that CCL18 can convert memory T-cells to Tregs [87]. Tregs, in turn, can upon coculture induce macrophages to display typical features of alternatively activated macrophages such as CD163 and CD206 and increased production of CCL18 [88], providing a positive feedback loop. Finally, as a CCR3 antagonist [89], CCL18 may limit the recruitment of eosinophils and basophils and hence dampen a local pro-allergic reaction [89, 90]. These data on the role of CCL18 under normal physiological conditions gave an indication that CCL18 might play a role in tumor development. This was underscored by the finding of high levels of expression of CCL18 by tumor-associated macrophages in glioma and ovarian and gastric cancer [91–94]. Furthermore, it was shown that the serum level of CCL18 was elevated in epithelial ovarian cancer patients. In fact, in a study of 51 patients with epithelial ovarian cancer, 27 patients with benign ovarian lesions and 29 healthy volunteers, serum CCL18 gave a sensitivity of 84.3% and a specificity of 91.1% [94]. As Duluc et al. [95] showed that IFN gamma was able to switch immunosuppressive TAM into immunostimulatory cells, with a concomitant reduction in CCL18 secretion, this may be a potential route for therapy.

Recently, PITPNM3 was identified as the functional receptor for CCL18 that mediates CCL18 effect and activates intracellular calcium signaling. This receptor is the mammalian homologue for *Drosophila melanogaster* rdgB, which is an essential protein for photoreceptor-cell survival and light response [96]. However, the protein appears to be also involved in regulation of cytoskeletal elements [96], which may provide a link to invasion and metastasis. In fact, it was

shown that suppression of PITPNM3 abrogated the effect of CCL18 on the invasion and metastasis of breast cancer xenografts [97]. This receptor might therefore be a potential target for therapy.

On the other hand, a tumor-suppressive function of CCL18 cannot be entirely ruled out as Leung et al. [92] reported that in gastric cancer, CCL18 was expressed by a subset of tumor-associated macrophages, located at the tumor invasion front and that high CCL18 expression levels were associated with prolonged overall and disease-free survival.

2.7. MMPs. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases, which function to degrade all kinds of extracellular matrix proteins. The MMPs have been shown to play important roles in tissue remodeling associated with various physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair, cirrhosis, arthritis, and metastasis. MMP-2 and MMP-9 especially are thought to be important in preparing the way for tumor cells to metastasize. In contrast, MMP-12 seems to have an antitumoral activity, in that it both retards tumor growth and suppresses growth of lung metastases [98]. Similarly, MMP-3 is thought to be expressed as a protective response and may play a role in host defense during tumorigenesis [99], although MMP3 has also been associated to vascular invasion by immunohistochemistry [100]. Furthermore, MMP-3 regulates macrophage secretion of prostaglandin E2 and expression of MMP-9 [101]. Specific endogenous tissue inhibitors of metalloproteinases (TIMPs) act to inhibit MMPs. These TIMPs comprise a family of four protease inhibitors: TIMP1, TIMP2, TIMP3, and TIMP4. It has been shown that in renal cell carcinoma the balance between MMP and TIMP is disturbed, possibly due to the production of radical oxygen species by TAM [102].

3. Treatment

3.1. Blocking the Di erentiation and Recruitment of Macrophages. Although the association between CSF-1 and enhanced tumorigenesis is evident, CSF-1 also plays an important role in lactation, ovulation, preimplantation, and placental function [103, 104], restricting its role as therapeutic target. The expression of c-fms in normal tissue, on the other hand, is limited to macrophages, except during pregnancy [105], making it a better target for therapy, although indiscriminate destruction of macrophages can have serious consequences for health, including decreased liver function and vulnerability to infectious diseases. Nevertheless, a number of agents have been developed to specifically target c-fms, as well as some multitargeted agents, showing c-fms inhibition in enzyme and cell-based assays [106]. Currently, three phase 1 clinical trials involving c-fms inhibitors are recruiting patients (NCT01004861, NCT01316822, and NCT01346358) (clinicaltrials.gov, accessed 2011/08/26). These studies will show whether c-fms inhibitors are of value in cancer therapy or result in unacceptable levels of toxicity.

The minor groove binding agent Yondelis was used to investigate the immunomodulatory effects on leukocytes. At subcytotoxic concentrations, Yondelis inhibited the differentiation of monocytes to macrophages. The production of CCL2 and IL6 by monocytes, macrophages, TAMs, and tumor cells was also markedly reduced [107]. In the case of human myxoid liposarcoma, *in vitro* treatment of primary tumor cultures and/or cell lines with noncytotoxic concentrations of Yondelis selectively inhibited the production of CCL2, IL-6, and VEGF. A xenograft mouse model of human MLS showed marked reduction of CCL2, CD68+-infiltrating macrophages, and CD31+ tumor vessels after treatment with Yondelis. Similar findings were observed in a patient tumor sample excised after several cycles of therapy [108].

After subcutaneous injection of prostate cancer cells in male SCID mice, systemic administration of anti-CCL2 antibodies significantly retarded tumor growth and attenuated macrophage infiltration, with a concomitant decrease in microvascular density [109]. Treatment of immunodeficient mice bearing human breast cancer cells with a neutralizing antibody to CCL2 resulted in a significant decrease of macrophage infiltration, angiogenic activity, and tumor growth [68]. Similarly, CCL2 blockade by antimurine CCL2 monoclonal antibodies significantly slowed the growth of primary tumors and inhibited lung metastasis in animal models of non-small-cell lung cancer. The treatment did not have effect on the number of TAM, but seemed to elicit a change of TAM to a more antitumor phenotype [110]. To investigate another route to block CCL2, a dominant negative CCL2 mutant gene was transfected in the thigh muscle in a model of human melanoma cells being implanted onto the back of a mouse. The dominant negative CCL2 inhibited TAM recruitment and partially reduced tumor angiogenesis and tumor growth [111].

CNTO 888, a human mAb specific for human CCL-2, is under current investigation in two clinical trials, one as single agent in patients with metastatic prostate cancer (NCT00992186) and the other in combination with standard of care chemotherapy in patients with solid tumors (NCT01204996). Furthermore, MLN1202, a highly specific humanized monoclonal antibody that interacts with CCR2 and inhibits CCL-2 binding, is being used in a phase II trial in patients with bone metastases (NCT01015560). In a related study on MLN1202 treatment in patients at risk of atherosclerotic cardiovascular disease, patients were genotyped for the 2518 A → G polymorphism in the promoter of the MCP-1 gene. Patients with A/G or G/G genotypes in the MCP-1 promoter had significantly greater reductions in high-sensitivity C-reactive protein levels than patients with the wild-type A/A genotype [112]. This polymorphism may also affect the outcome in studies of cancer patients.

Following the initial report on cyclooxygenase-2 (COX-2) overexpression in colorectal cancer [113], COX-2 has been the focus of attention as a potential target for cancer treatment. In contrast with COX-1, which is constitutively expressed, COX-2 expression levels are low or undetectable in normal tissues under basal conditions, with the exception of the seminal vesicles, kidneys, and certain areas of the brain, and expression levels increase transiently upon stimulation

[114]. However, COX-2 overexpression has been found in a wide range of solid and hematological tumors (reviewed in [115]). Clinical and epidemiological investigations as well as experimental studies have shown that COX-2 contributes to tumourigenesis in every stage: tumor initiation, tumor promotion, and tumor spread. One of the mechanisms involved is the creation of an inflammatory environment. As discussed in Section 1, chronic inflammation constitutes a risk factor for carcinogenesis. Prostaglandin E2 (PGE2) is the most abundant among the prostaglandins produced by COX-2-expressing tumors [116]. The release of PGE2 provides a positive feedback loop [117], which ensures lasting levels of COX-2 in the tumor environment. A role of PGE2-dependent signaling pathways has been described in tumor growth, angiogenesis, tumor invasion and metastasis, tumor survival, and tumor immune tolerance (reviewed in [115]). Given the importance of COX-2 and PGE2, a range of COX-2 inhibitors have been developed. These compounds showed encouraging results *in vitro* and *in vivo* [118, 119] and were introduced in clinical trials for both chemoprevention as well as cancer therapy. Three large randomized clinical trials confirmed the efficacy of COX-2 inhibitors for chemoprevention [120–122], however, at the cost of a significant increase in incidence and severity of thrombotic events [123]. This increased risk, however, could not be confirmed in a meta-analysis of 72 studies, unless patients had previous risk factors for cardiovascular disease [124].

More specifically, towards a potential association between COX-2 and TAM, the COX inhibitor DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone) was investigated in a rat tumor model and significantly reduced the CCL2 production, as measured both in tumor tissue and in the systemic circulation, with concomitant reduction of the tumor size [125]. Despite these, and other encouraging preclinical results, results of large randomized trials, comparing chemotherapy alone or in combination with COX inhibitors, have been less promising so far [126, 127].

3.2. Killing of Macrophages in the Tumor Microenvironment. Bisphosphonates are known to kill macrophages. In a study by Gazzaniga et al., clonodrate-loaded liposomes (CLIPs) were administered to melanoma-bearing mice. The macrophage depletion following this treatment resulted in smaller tumors, with fewer vascular structures [128]. Similarly, in an orthotopic, immunocompetent murine model of diffuse malignant peritoneal mesothelioma, intraperitoneal injection of CLIP leads to apoptosis in tumor cells. Furthermore, when CLIP was injected together with mesothelioma cells, there were a 4-fold reduction in number of tumors and a 5-fold reduction in invasion and metastasis, compared to liposome-encapsulated PBS. Even in mice bearing established tumors, i.p. injected CLIP resulted in a significant reduction in number of tumors [129]. In a study investigating the use of CLIP in several types of tumor in mice, Takahashi et al. showed that injection of CLIP in four spots around the tumor on day 0 or 5 after tumor injection and every third day thereafter resulted in tumor rejection after 12 injections. Depletion of macrophages by CLIP injection before radiotherapy increased the antitumor effect of ionizing radiation [130]. The combination of CLIP with the small molecule sorafenib, for the treatment of a mouse metastatic liver cancer model, was shown to inhibit tumor progression, tumor angiogenesis, and the development of lung metastasis significantly better than sorafenib alone [131]. In this study, zoledronic acid, another bisphosphonate, was shown to be even more effective than clodronate [131]. In an *in vitro* model of prostate cancer cell-macrophage interaction, zoledronic acid selectively suppressed the expression of MMP-9 by TAM, whereas the expression of other mediators was not lowered. Zoledronic acid also boosted the production of type-1 cytokines by PC-TAM in response to immunomodulators such as IL-12, which is known to polarize macrophages towards an antitumoral M1 phenotype [132]. In conclusion, depletion of macrophages in and around the tumor has been shown to give encouraging results in mouse models, most likely by taking out the paracrine signaling by TAM to tumor cells. However, in most cases, bisphosphonates were given simultaneously with the challenge with tumor cells, which obviously is not the situation in patients. Nevertheless, bisphosphonates have been used extensively in humans, while it becomes apparent that their usage is not without risk. The most common adverse effects associated with the use of bisphosphonates are renal toxicity, acute-phase reactions, gastrointestinal toxicity, and osteonecrosis of the jaw. The incidence of these adverse events varies significantly among bisphosphonates. Renal toxicity is a potentially life-threatening event reported in studies of zoledronic acid and, to a lesser extent, pamidronate. In contrast, the renal safety profile of intravenous ibandronate and oral bisphosphonates is similar to that of placebo. Acute-phase reactions occur only with intravenous aminobisphosphonates and may be more common with zoledronic acid. Gastrointestinal effects occur only with oral agents (clodronate and ibandronate) [133]. Careful monitoring of patients, not only for the adverse events described above, but also for infectious diseases and liver failure, due to the indiscriminate destruction of all phagocytic myeloid cells by bisphosphonates, is strictly necessary.

3.3. Repolarization of TAMs. Administration of the proton pump inhibitor pantoprazole to mice with T-cell lymphoma resulted in enhanced TAM recruitment to the tumor environment. These TAMs had the M1 phenotype. Pantoprazole leads to a reversal of immunosuppression and a shift in the cytokine profile [134]. The antitumor effect of pantoprazole was evaluated *in vivo* by a xenograft model of nude mice. After pantoprazole treatment, apoptotic cell death was seen selectively in cancer cells. By contrast, normal gastric mucosal cells showed resistance to pantoprazole-induced apoptosis through the overexpression of antiapoptotic regulators including HSP70 and HSP27 [135]. A phase I study evaluating pantoprazole in combination with doxorubicin

for advanced cancer patients is currently recruiting patients (NCT01163903).

IL12, which promotes tumoricidal responses and is normally produced by M1 macrophages, induces tumor regression when used in tumor-bearing mice [136]. This treatment induced a reduction of M2-associated chemokines and an increase in M1-associated chemokines [136]. Further study by this group revealed that the rapid release of IL-15 after IL-12 treatment is essential for infiltration of the tumor and surrounding tissue by leukocytes, including CD8+ T cells, substantiating the repolarization by IL-12 to M1 [137]. In a study by Airoldi et al., the IL-12 receptor beta2 unit was introduced into Calu6 cells by transfection. IL-12 treatment of transfected Calu6/beta2(+) cells inhibited angiogenesis *in vitro*. Tumors in SCID/NOD mice, formed by cells transfected with IL-12Rbeta2, were significantly smaller following IL-12 versus PBS treatment due to inhibition of angiogenesis and of IL-6 and VEGF-C production [138]. Application of repeated doses of IL-12 to cancer patients resulted in a Th1 to Th2 shift (increase in IL10, decrease in IFN-gamma, TNF-alpha, and IP10 in serum of the patients) [139, 140]. This may indicate a potential limitation of the use of IL-12 as a single agent, which is underscored by the finding of a limited efficacy in most clinical trials with IL-12 [141]. However, combined administration of IL-12 with other cytokines, such as IL-2, IL-15, IL-7, IL-21, IL-18, GM-CSF, or IFN-alpha, seems to overcome this problem [142]. Furthermore, when coadministered, a lower effective dose of IL-12 is necessary, reducing potential toxicity, as high toxicity is another limitation of IL-12 therapy [143]. Finally, local administration of the cytokine(s), rather than systemic administration, also reduces the problem of toxicity [142]. For polarization of macrophages towards the alternative phenotype (M2), NF-κb needs to be active. When NF-κb signaling is inhibited, the macrophages become cytotoxic to tumor cells, resulting *in vivo* in regression of advanced tumors [144]. Inhibition of NF-κb signaling may therefore be an alternative for IL-12 administration.

The host-produced histidine-rich glycoprotein (HRG) was shown to inhibit tumor growth and metastasis while improving sensitivity to chemotherapy. This was accomplished by skewing TAM polarization from M2 to M1 phenotype, accompanied by a promotion of antitumor immune responses and vessel normalization, through downregulation of the placental growth factor [145]. The RCAS/TV-A mouse model for gliomas was used to investigate the effect of HRG on brain tumor development. Tumors were induced with platelet-derived growth factor-B (PDGF-B), in the presence or absence of HRG. HRG was found to have little effect on tumor incidence but could significantly inhibit the development of malignant glioma and completely prevent the occurrence of glioblastoma [146].

3.4. Inhibition of M2 Macrophage Functions. Prednisolone has been used to investigate its effect on TAM melanoma-bearing mice. The major inhibitory action on tumor growth was the reduction of TAM-mediated production of proangiogenic factors, whereas the production of antiangiogenic factors was hardly affected [147]. Liposomes encapsulating prednisolone phosphate were developed to evaluate the local delivery of liposomal glucocorticoids to the tumor and its importance for the therapeutic response. A single dose of prednisolone liposomes was found to significantly inhibit tumor growth in mice, subcutaneously inoculated with B16F10 melanoma cells. Uptake of liposomes by TAM was limited to only 5% of the TAM population, and the therapy did not lead to TAM depletion. However, a 90% drop in white blood cell count after prednisolone administration was observed. This depletion may reduce tumor infiltration of monocytes, which stimulate angiogenesis, and possibly cocontributes to the antitumor effects [148].

Silibinin has demonstrated anticancer effects against, amongst others, human prostate adenocarcinoma cells [149], human ovarian cancer [150], human colon cancer cells [151], and human lung carcinoma cells [152]. Oral silibinin was tested on established lung adenocarcinomas in A/J mice. Silibinin strongly decreased tumor number and size, probably by an antiangiogenic mechanism [153]. One clinical study using silibinin in advanced hepatocellular carcinoma is ongoing (NCT01129570), and another study in men with prostate cancer has been completed [154, 155]. This study showed that high-dose oral silybin-phytosome achieved high blood concentrations transiently, but only low levels of silibinin were seen in prostate tissue. Furthermore, one of the six treated patients developed a grade 4 postoperative thromboembolic event [155].

In the FL-2000 trial, patients with follicular lymphoma were randomly assigned to receive standard treatment (cyclophosphamide, doxorubicin, etoposide, prednisolone, and interferon) or standard treatment plus rituximab. This chimeric monoclonal antibody binds to CD20, which is widely expressed on B cells, from early pre-B cells to later in differentiation. In the control arm, a low number of TAM (CD68+) was associated with a better event-free survival, whereas this effect was not observed in the rituximab arm, which suggests that rituximab is able to circumvent the unfavorable outcome associated with a high number of TAM [156]. In fact, after rituximab and cyclophosphamide-doxorubicin-etoposide-prednisone regimen, high TAM content correlated with longer survival rates. In multivariate analyses, TAM content remained an independent prognostic factor for OS and PFS [157]. It was recently shown that, *in vitro*, Ms4a8a mRNA and MS4A8A protein (a CD20 homologue) expression was strongly induced in bone-marrow-derived macrophages by combining M2 mediators (IL-4, glucocorticoids) and tumor-conditioned media [158]. If this CD20 homologue is also expressed on TAM, this could explain the activity of rituximab.

4. Conclusions

It is clear that there are several instants of interaction between tumor cells and macrophages where therapeutic intervention is a possibility. On the other hand, early stages of interaction, such as differentiation and chemotaxis, may already have occurred at the time of diagnosis. Whereas in mouse models c-fms inhibitors, anti-CCL2 monoclonal antibodies, or bisphosphonates can be given before, or simultaneously,

with inoculation with tumor cells, in humans this is not the case. Limited evidence is available to support posthoc efficacy of these kinds of treatment.

Perhaps the most interesting intervention would be the repolarization of macrophages, as this will turn the ally into an enemy, fighting the cancer at close range. From the agents described to invoke repolarization, IL-12 might be the most interesting candidate, with 66 clinical trials in different stages of execution. While not designed to investigate the effect of IL-12 on the interaction between tumor and TAM, these studies may reveal positive effects that will pave the way for new studies investigating the effect of IL-12 on TAM, specifically.

Regardless of the route chosen to block interaction between tumor cells and macrophages, it has become clear that whereas a reduction in tumor growth, angiogenesis, and metastasis can be obtained, complete clearance of the tumor is unlikely. Therefore, combination with chemo- and/or radiotherapy will remain essential.

Disclosure

Anja Brouwer participated in this work as part of her Masters degree in Medicine.

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Review Article

Conversion of Tumors into Autologous Vaccines by Intratumoral Injection of α-Gal Glycolipids that Induce Anti-Gal/α-Gal Epitope Interaction

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Anti-Gal is the most abundant antibody in humans, constituting 1% of immunoglobulins. Anti-Gal binds specifically α-gal epitopes (Galα1-3Galβ1-4GlcNAc-R). Immunogenicity of autologous tumor associated antigens (TAA) is greatly increased by manipulating tumor cells to express α-gal epitopes and bind anti-Gal. Glycolipids with α-gal epitopes (α-gal glycolipids) injected into tumors insert into the tumor cell membrane. Anti-Gal binding to the multiple α-gal epitopes *de novo* presented on the tumor cells results in targeting of these cells to APC via the interaction between the Fc portion of the bound anti-Gal and Fcγ receptors on APC. The APC process and present immunogenic TAA peptides and thus, effectively activate tumor specific CD4+ helper T cells and CD8+ cytotoxic T cells which destroy tumor cells in micrometastases. The induced immune response is potent enough to overcome immunosuppression by Treg cells. A phase I clinical trial indicated that α-gal glycolipid treatment has no adverse effects. In addition to achieving destruction of micrometastases in cancer patients with advance disease, α-gal glycolipid treatment may be effective as neo-adjuvant immunotherapy. Injection of α-gal glycolipids into primary tumors few weeks prior to resection can induce a protective immune response capable of destroying micrometastases expressing autologous TAA, long after primary tumor resection.

1. Introduction

Destruction of detectable metastases by resection or ablation may prolong survival, but it does not affect invisible micrometastases which can develop into lethal metastases. Moreover, it is likely that in a large proportion of patients treated by novel targeted therapies, micrometastases may not be completely eliminated. This is since there is a high probability that some metastatic cells residing far enough from capillaries will not be susceptible to the therapeutic effect of drugs because of diminishing concentration of drugs diffusing from the nearby capillary. A lasting antitumor protection that effectively destroys micrometastases may be achieved by immunotherapy that stimulates the immune system to react against the multiple tumor-associated antigens (TAAs). This principle is demonstrated in the life-long protection against EBV transformed polyclonal B cells which are "kept at bay" by EBV-specific T cells. Immunosuppression (as in allograft recipients) may result in appearance of such transformed B cells as multiclonal lymphomas due to the suppression of protective T cell activity [1]. The protective effect of the human immune system against TAA on tumor cells is further illustrated by the high correlation between the extent of T cell infiltration within tumors and positive prognosis reported in melanoma, ovarian cancer, colorectal carcinoma, and other tumors [2–6]. In particular, detailed studies of Galon and colleagues demonstrated a distinct inverse correlation between the density of infiltrating CD8+ and Th1 memory T cells in resected colorectal carcinoma and the relapse of the disease [4–6]. The observed exclusive infiltration of T cells into tumors without affecting normal tissues implies that the observed antitumor immune response is aimed specifically against antigens (Ags) on tumor cells, that is, against TAA. This further implies that

eliciting an immune response against these Ags may result in immune protection against the tumor cells expressing them.

2. Autologous TAA as Antitumor Vaccine

TAA can be common to a given tumor in many patients having the same type of tumor, or can be unique to the individual patient. Immunotherapy against known common TAA has been unsatisfactory, possibly since such TAA are of weak immunogenicity due to their presence in low amounts both on normal cells and on embryonic cells [7, 8]. Unique TAA appears due to genomic instability in tumor cells and are generated by multiple coding mutations which differ from one patient to the other. Many of these mutations result in small changes in proteins that may provide advantageous growth to tumor cells [9–13]. Other mutations are neutral since they do not affect the structure or function of the mutated protein. The mutated proteins can function as autologous TAA that may elicit a protective antitumor immune response, since they are present in tumor cells and absent in normal cells. Such a protective immune response against autologous TAA may be beneficial in achieving immune protection against metastatic tumor cells. The immune system is capable of detecting and reacting against small changes that give rise to autologous TAA. This can be inferred from the extensive immune response to blood group Ags where the difference of the small N-acetyl group on the terminal galactosyl in blood group A, in comparison to blood group B (i.e., GalNAcα1-3Gal and Galα1-3Gal, resp.) is sufficient for inducing the production of anti-A antibody (Ab) in blood group B individuals.

Characterization and production of the multiple autologous TAA in each individual patient for vaccinating purpose is not feasible at present. Thus, the tumor itself is the only current practical source for autologous TAA [14]. As with any microbial vaccine, an effective immune response against autologous TAA requires effective uptake of the tumor cells and cell membranes by antigen presenting cells (APCs) such as dendritic cells and macrophages. The internalized TAA are transported by APC to the regional lymph nodes, processed by these cells, and presented on MHC class I and class II molecules as TAA peptides that activate tumor-specific CD8+ cytotoxic T cells and CD4+ Th1 cells, respectively [15, 16]. However, vaccination with unprocessed tumor cells obtained from the patient is usually ineffective since tumor cells evolve to evade recognition by APC [7–10] and thus are "ignored" by the immune system. This is clearly indicated by the ability of tumor cells to reside in lymph nodes without being affected by the immune system. Moreover, TAA are usually "concealed" from the immune system because the tumor cytokine milieu is often suppressive toward immune function and induces tolerance, anergy, or lymphocyte death [17–19]. Thus, studies have been aimed to recruit APC into the lesions by intratumoral administration of immunomodulators such as GM-CSF or CpG oligonucleotides [19, 20]. Although effectively recruited, the APC cannot identify tumor cells within the lesion as cells that "ought" to be internalized, since the tumor cells lack identifying markers that label them for uptake by APC. Thus, uptake of tumor cells by recruited APC is suboptimal as it is mediated by random endocytosis [14].

It is well established that one of the most effective mechanisms by which APC as macrophages and dendritic cells can internalize vaccinating Ags and process them for T cell activation is the formation of immune complexes with the microbial and tumor vaccinating Ags. The interaction of the Fc portion of the immunocomplexed Ab molecules with Fcγ receptors (FcγRs) on APC generates signals for Ag internalization as well as for maturation of dendritic cells internalizing the Ag and subsequent effective stimulation of the immune system [21–30]. Targeting tumor cells via Fc/FcγR interaction to macrophages and dendritic cells enables these APC to internalize TAA of the tumor cells coated with an IgG Ab. Such targeting is feasible in all humans that are not severely immunocompromized by exploiting the natural anti-Gal Ab and its ligand the α-gal epitope.

3. The Natural Anti-Gal Antibody and The α-gal Epitope

Anti-Gal is the most abundant natural Ab in human blood, constituting ~1% of serum immunoglobulins [31]. It is produced throughout life in response to antigenic stimulation by gastrointestinal bacteria [32]. Anti-Gal binds specifically to the α-gal epitope. This epitope has the structure Galα1-3Galβ1-4GlcNAc-R [33, 34]. The α-gal epitope is present on cell surface glycolipids and glycoproteins of nonprimate mammals, prosimians, and New World monkeys [35–37]. The α-gal epitope is synthesized on carbohydrate chains of glycolipids and glycoproteins in mammalian cells by the glycosylation enzyme α1,3galactosyltransferase (α1,3GT) [36, 38]. Because of immune tolerance, mammalian species producing α-gal epitopes lack the natural anti-Gal Ab. In contrast, humans, apes, and Old World monkeys lack the α-gal epitope due to inactivation of the α1,3GT (*Ggta1*) gene in ancestral primates, and all produce the natural anti-Gal antibody [35–41].

Since anti-Gal is present in large amounts in all immunocompetent humans and Old World monkeys, administered α-gal epitopes form *in situ* immune complexes with it. One area demonstrating this Ag/Ab interaction has been xenotransplantation in which pig cells or pig organs are transplanted into humans or monkeys. Anti-Gal binding to the multiple α-gal epitopes on cells of pig xenografts causes the rapid rejection of such xenografts (e.g., pig heart or kidney) in humans, or in monkeys, by complement-dependent cytotoxicity (CDC) and by antibody-dependent cell cytotoxicity (ADCC) [42–45]. As described below, injection of α-gal glycolipids into tumors in a mouse experimental model producing anti-Gal Ab results in expression of α-gal epitopes on tumor cells within the injected lesion, in a manner similar to the expression of these epitopes on pig cells. The subsequent binding of anti-Gal Ab to the α-gal epitopes *de novo* expressed on the tumor cells results in destruction of tumor cells as in xenograft rejection and the targeting of tumor cells and cell membranes to APC.

4. Anti-Gal-Mediated Targeting of Ags to APC and Increased Immunogenicity in the OVA model

The principle of anti-Gal-mediated increased immunogenicity of vaccines by formation of immune complexes with α-gal epitopes could be illustrated with hen egg ovalbumin (OVA) as the immunizing Ag [46]. OVA serves as an effective model for an immunizing Ag since there are several highly sensitive immunological tools that enable evaluation of the internalization and processing of this Ag in APC and presentation of its most immunogenic peptide SIINFEKL on class I MHC molecules. Furthermore, activation of CD8+ T cells can be evaluated following the specific interaction with SIINFEKL when presented on class I MHC molecules. The immune response was analyzed in the experimental model of α1,3GT knockout mice (KO mice) which lack α-gal epitopes and produce the anti-Gal Ab [47]. OVA was encapsulated within liposomes that express multiple α-gal epitopes (referred to as α-gal liposomes) [46, 48, 49]. Uptaking and processing of OVA encapsulated within α-gal liposomes by KO mouse APC was found to be several fold higher when the liposomes bound anti-Gal than in the absence of this Ab. This increased uptake was due to Fc/Fcγ R interaction between the Fc portion of anti-Gal on liposomes and Fcγ R on APC [46]. APC in draining lymph nodes displayed 5–8 fold higher presentation of SIINFEKL than APC in draining lymph nodes of mice lacking anti-Gal. Accordingly, the activation of SIINFEKL specific T cells as measured by intracellular staining for IFNγ production and by binding of tetramers carrying SIINFEKL was 2–6 fold higher in the presence of anti-Gal in the immunized mice than in the absence of this targeting Ab [46]. In addition, cytolytic activity of SIINFEKL-specific T cells was ∼8 fold higher and the titer of anti-OVA Abs was 32 fold higher in vaccinated mice that had the anti-Gal Ab than in mice lacking this Ab. These studies confirmed the hypothesis that anti-Gal binding to vaccinating Ags presenting α-gal epitopes induces effective uptake and processing of the Ag by APC and increased transport to draining lymph nodes. In the lymph nodes the immunogenic peptides presented by APC induce a markedly higher activation of CD8+ and CD4+ T cells than in the absence of this mechanism [46]. This conclusion is further supported by observations on the immune response to influenza virus vaccine and to gp120 of HIV following anti-Gal-mediated targeting to APC. Inactivated influenza virus processed enzymatically to express α-gal epitopes elicited a 100-fold higher specific CD4+ T cell response and anti-influenza Ab response and ∼5 fold higher CD8+ T cell response than vaccinating virus lacking α-gal epitopes [50]. Accordingly, KO mice immunized with virus vaccine presenting α-gal epitopes were ∼11 fold more resistant to challenge with live virus than KO mice immunized with virus lacking α-gal epitopes [50]. Similarly, gp120 envelop glycoprotein of HIV processed to express α-gal epitopes was found to elicit in KO mice ∼ 100 fold higher T cell response (determined by ELISPOT) and anti-gp120 Ab response (determined by ELISA) than gp120 lacking α-gal epitopes [51]. All these studies in KO mice suggested that manipulation of autologous tumor cells in cancer patients to express α-gal epitopes may result in conversion of such cells into effective vaccines that elicit a protective immune response against autologous TAA because of their effective anti-Gal-mediated targeting to APC.

5. Increased Immunogenicity Following Anti-Gal-Mediated Uptake of Tumor Cells by APC

Studies on increasing immunogenicity of TAA by anti-Gal-mediated targeting of tumor cells to APC were initially performed with the mouse melanoma B16 cell line, lacking α-gal epitopes, and processed to express these epitopes by stable transfection with the α1,3GT gene. These cells were irradiated and injected into anti-Gal producing KO mice [52–54]. Vaccination with irradiated B16 cells expressing α-gal epitopes was found to elicit a protective immune response that prevented tumor growth following challenge with live tumor cells lacking this epitope [52, 54]. Such protection was also found in mice challenged with live melanoma cells prior to the immunization with the irradiated tumor cells expressing the α-gal epitopes [53]. Recently, a similar protective effect of tumor cells expressing α-gal epitopes has been demonstrated in a pancreatic adenocarcinoma model in KO mice [55]. The increased immunogenicity of TAA in both melanoma and pancreatic adenocarcinoma models was shown to be associated with effective anti-Gal-mediated uptake of the vaccinating cells by APC [52–55].

The method of genetic manipulation by stable transfection with α1,3GT gene is effective in inducing α-gal epitope expression in cell lines since it requires cell division for the insertion of this gene into the DNA of the tumor cells. Because of the need for extensive cell division, this genetic manipulation is not effective *in vivo* in solid tumors that are injected with a vector containing the α1,3GT gene. Introduction of the α1,3GT gene into tumor cells and transient expression of α-gal epitopes was also found to be effective in cell lines using an adenovirus vector containing this gene [56]. Accordingly, immunization with B16 melanoma cells transduced with adenovirus containing the α1,3GT gene was found to elicit immune protection against the tumor challenge [56]. However, injection of this vector into tumor lesions resulted in a very limited expression of α-gal epitopes because of poor diffusion of the injected virus beyond the injection area (unpublished observations). An alternative method has been developed for *in vivo* expression of α-gal epitopes on autologous tumor cells which consists of intratumoral injection of α-gal glycolipids.

6. α-Gal Glycolipids Insert α-gal Epitopes into the Membrane of Tumor Cells within Treated Lesions

α-Gal glycolipids are glycolipids consisting of a ceramide lipid tail and a carbohydrate chain with one or several carbohydrate branches (antennae), all of which are capped with the α-gal epitope [57]. A representative α-gal glycolipid

with 10 carbohydrate units (ceramide decahexoside) and two branches is illustrated in Figure 1(a). The number of branches carrying α-gal epitopes can be 1–5, or more, all having the α-gal epitope as the terminal carbohydrate structure. Rabbit red blood cells (RBCs) were found to be a very rich source of α-gal glycolipids. These RBC have on their membrane α-gal glycolipids with 5 carbohydrates and with longer chains that increase in size by increment of 5 carbohydrate (i.e., 10, 15, 20, and up to 40 carbohydrates (with the exception of a glycolipid with 7 carbohydrates)) [58–63]. Each increment also generates a new branch capped with α-gal epitopes [58–63]. α-Gal glycolipids are extracted from rabbit RBC membranes by their incubation in a mixture of chloroform and methanol [57]. The hydrophilic glycolipids are further separated from the hydrophobic phospholipids and cholesterol by a process called Folch partition [64]. Extracted α-gal glycolipids dissolve in water or PBS in a ball-like forms called micelles in which the hydrophobic portion of the glycolipid (i.e., the lipid tail) is in the core of the micelle whereas the hydrophilic carbohydrate chain protrudes into the aqueous surrounding.

Incubation of α-gal glycolipid micelles with tumor cells for 2 h at 37°C results in spontaneous insertion of these glycolipids into the tumor cell membranes. This is since the hydrophobic lipid tail of the α-gal glycolipid is energetically much more stable when surrounded by the phospholipids of the lipid bilayer in the cell membrane than when it is surrounded by water molecules in the micelle. The insertion of α-gal glycolipids into the tumor cell membranes results in presentation of multiple α-gal epitopes on tumor cells. These epitopes protrude from the tumor cell membrane and readily bind the anti-Gal Ab (Figure 1(b)) [57]. The effective *in vitro* insertion α-gal glycolipids into B16 melanoma cells [57] and into human tumor cells [65] suggested that a similar insertion may occur *in vivo* in tumor lesions injected with these glycolipids. Effective *in vivo* insertion into a large proportion of the tumor cells within the lesion is achieved by injection in several regions of the tumor. It should be stressed that this insertion is not selective and occurs in both malignant and normal cells in the lesion. This *in vivo* insertion could be visualized in B16 melanoma lesions by staining with a lectin specific for α-gal epitopes (*Bandeiraea (Gri onia) simplicifolia IB*$_4$) [57]. Injection of α-gal glycolipid micelles into tumor lesions is likely to result in several processes includingthe following.

6.1. Recruitment of APC by Complement Cleavage Chemotactic Factors. Anti-Gal/α-gal epitope interaction activates the complement system and generates chemotactic complement cleavage peptides such as C3a and C5a. These chemotactic factors induce an extensive recruitment of APC such as macrophages and dendritic cells into the treated lesion. Thus, this treatment enables the immune system to overcome the immunosuppressive conditions within solid tumor lesions, induced by microenvironment and local cytokine milieu and by regulatory T (Treg) cells [17–19, 65].

6.2. Insertion of α -gal Glycolipids into Tumor Cell Membranes. α-Gal glycolipids injected as micelles into lesions spontaneously insert into the tumor cell membranes, resulting in the presentation of multiple α-gal epitopes on the membrane of the tumor cells (Figure 1(b)).

6.3. Destruction of Tumor Cells by Anti-Gal Binding to α-gal Epitopes on the Cells. Anti-Gal binding to α-gal epitopes of the inserted glycolipids mediates tumor cell destruction in a process similar to xenograft rejection. Bound anti-Gal IgM molecules activate complement and induce cell lysis by complement-dependent cytotoxicity (CDC) [57]. Binding of anti-Gal IgG molecules to α-gal epitopes on cells further facilitates Ab-dependent cell-mediated cytolysis of the cells (ADCC) [42].

6.4. Targeting of Tumor Cells to APC for Their Conversion into Endogenous TAA Vaccine. Anti-Gal IgG molecules bound to α-gal epitopes on tumor cells in treated lesions further bind via their Fc portion to Fcγ R on dendritic cells and macrophages and stimulate these APC to internalize the opsonized tumor cells and cell membranes with the autologous TAA (Figure 1(b)). The internalized TAA, transported by the APC to draining lymph nodes, are processed and the immunogenic TAA peptides presented for the activation of tumor-specific T cells. These activated T cells leave the lymph nodes a circulate in order to seek and destroy tumor cells in micrometastases which express the immunizing TAA.

7. Treatment of Mouse Melanoma by Intratumoral Injection of α-gal Glycolipids Prevents Tumor Growth

Evaluation of α-gal glycolipid immunotherapy was performed in the model of KO-mice-bearing syngeneic cutaneous B16 melanoma [57, 65]. Melanoma lesions with a size of ∼5 mm were formed within one week after subcutaneous injection of 10^6 melanoma cells of the cell line B16. Injection of 1 mg α-gal glycolipids into such lesions resulted in *in situ* insertion of these glycolipids into tumor cell membranes which could be demonstrated by immunostaining of tumor sections with *Bandeiraea simplicifolia* IB4 lectin which binds specifically to α-gal epitopes [57]. The interaction between α-gal glycolipids injected into B16 melanoma lesions of KO mice and the anti-Gal Ab further resulted in activation of the complement system and the formation of complement cleavage chemotactic factors that induced rapid recruitment of dendritic cells and macrophages. Thus, effective recruitment could be demonstrated already within 48 h after injection [57]. This recruitment further increases within 7 days, but it could not be observed in PBS-injected tumors, implying that in the absence of α-gal glycolipids, the immune system is "oblivious" to the growing tumor [57]. A similar rapid recruitment of macrophages was observed in KO mouse skin after injection of α-gal nanoparticles (submicroscopic liposomes) comprised of α-gal glycolipids, phospholipids, and cholesterol [49]. This recruitment was inhibited by cobra venom factor which blocks activation of the complement system thereby preventing generation of complement chemotactic factors [49].

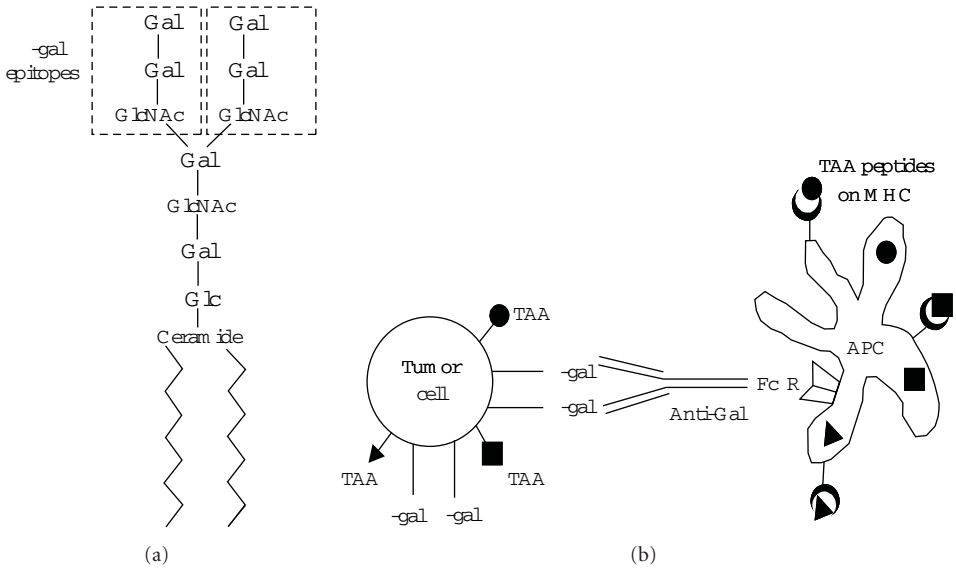

Figure 1: (a) Ceramide decahexoside as a representative α-gal glycolipid. This glycolipid has 10 carbohydrate branched chains. The α-gal epitope (Galα1-3Galβ1-4GlcNAc-R) marked by the broken line rectangles caps both carbohydrate branches. The terminal α-galactosyl (Gal) unit is linked α1,3 to the penultimate Gal of the carbohydrate chain by the glycosylation enzyme α1,3galactosyltransferase (α1,3GT). The lipid portion of α-gal glycolipids (ceramide) anchors the carbohydrate portion in the cell membrane via the two fatty acid tails. (b) Anti-Gal-mediated targeting of tumor cells to APC in lesions injected with α-gal glycolipids. Intratumoral injection of α-gal glycolipids results in insertion of these glycolipids in tumor cell membranes. Anti-Gal IgG binds *in situ* to α-gal epitopes on the inserted glycolipids. Subsequent interaction between the Fc portion of the bound anti-Gal and FcγR on the APC (illustrated as a dendritic cell) induces uptake of intact or lysed tumor cells by APC, resulting in effective internalization of the tumor-associated antigens (TAAs). Internalized TAA are processed and various immunogenic TAA peptides (, ,) are presented by the APC in association with class I and class II MHC molecules. These immunogenic peptides can activate tumor specific cytotoxic and helper T cells and elicit a protective antitumor immune response.

B16 melanoma is a very aggressive tumor that usually doubles its size in KO mouse skin every 4–8 days. However, the majority (65%) of melanoma lesions injected with α-gal glycolipids displayed no additional growth or regression in lesion size [57]. The remaining tumors displayed slower growth than control lesions injected with PBS (all of which did not stop growing). *In vitro* analysis of anti-Gal-mediated killing of B16 melanoma cells presenting α-gal epitopes indicated that both complement depended cytolysis and ADCC contribute to the destruction of tumor lesions injected [57]. A similar complement-mediated cytolysis by anti-Gal was observed in human melanoma cells that were incubated with α-gal glycolipids, then incubated with human serum containing anti-Gal Ab and complement [65].

8. Melanoma Lesions Injected with α-gal Glycolipids Are Converted into Autologous Vaccines

Similar to the lack of immune response against tumors in patients with advanced disease, there is no protective immune response in KO mice against untreated B16 tumors. This is indicated by the complete lack of APC infiltration in B16 melanoma tumors injected with PBS [57]. Moreover, melanoma lesions ablated by intratumoral injection of ethanol elicit no protective immune response against distant untreated lesions [65]. However, intratumoral injection of α-gal glycolipids converts the treated lesion into an endogenous autologous tumor vaccine which elicits an immune response against autologous melanoma-associated antigens (MAAs) on the tumor cells. By using B16 melanoma cells producing OVA as a surrogate TAA and employing detection methods as those described above for α-gal liposomes containing OVA [46], it was possible to demonstrate effective *in vivo* uptake of the tumor cells by APC in lesions injected with α-gal glycolipids [57]. In addition, the subsequent transport and presentation of SIINFEKL (the immunodominant OVA peptide) in the draining lymph nodes was much higher in mice with α-gal glycolipid-injected tumors than in those with PBS-injected tumors, that is, the number of SIINFEKL presenting APC in the draining lymph node in the former group was much higher than in the latter. This, in turn, resulted in a much higher number of tumor-specific activated T cells which mediated a systemic protective antitumor immune response. These T cells could be demonstrated *in vitro* by determining the number of SIINFEKL-specific CD8+ T cells in mice with B16/OVA treated with α-gal glycolipids versus PBS and by demonstrating the marked increase in their cytolytic activity against cells presenting SIINFEKL on class I MHC molecules [57]. A similar increase in MAA-specific T cells was observed in B16-bearing mice treated with α-gal glycolipids versus PBS control by analysis of IFNγ secretion in ELISPOT following incubation with immunodominant MAA peptides of tyrosinase and gp100 [65].

The *in vivo* protective effect of α-gal glycolipids was determined by evaluation of distant tumor development after treatment. KO mice producing anti-Gal Ab and bearing B16 melanoma lesions received 3 weekly injections of 1 mg α-gal glycolipids. One day after the third injection, the mice were challenged in the contralateral flank with 5×10^5 B16 cells and subsequent tumor growth was monitored. The majority (65%) of treated mice displayed no tumor growth in the challenge site whereas the remaining mice displayed a slower tumor growth than PBS-injected tumors [57]. Another control group consisted of mice in which the primary tumor was ablated by intratumoral injection of ethanol, similar to tumor ablation in the clinical setting. Ablation by ethanol successfully destroyed treated tumors, however this treatment did not induce any protective immune response against challenge with B16 cells in the contralateral flank [65].

Immunotherapy with α-gal glycolipids was further evaluated for inducing a protective immune response against an established distant micrometastasis. A micrometastasis was simulated by subcutaneous inoculation in the left flank with 10^4B16 cells at the same time that the right flank was inoculated with 10^6 tumor cells. After 5-6 days, the tumor developing in the right flank reached a size of 5 mm ("primary" tumor) whereas in the left flank inoculation site the tumor was not visible at that time point and simulated an established distant micrometastasis. The visible primary tumor received two intratumoral injections of either PBS or of α-gal glycolipids. In mice injected with PBS the simulated distant micrometastasis developed into a 4–12 mm lesion within 20 days. However, in 50% of mice in which primary tumors were injected with α-gal glycolipids, no lesions developed from the micrometastasis during the 30 days monitoring period. In the remaining mice, the simulated micrometastases developed, however, at a slower rate than that in the PBS-treated mice [65]. Overall, the prevention of tumor growth in the contralateral flank following injection of the primary tumor with α-gal glycolipids reflects the induction of a protective immune response against autologous MAA due to the conversion of the injected tumors into autologous vaccine. This further suggests that a similar treatment in humans may elicit an immune response capable of destroying micrometastases, thereby preventing them from developing into lethal metastases.

9. α-Gal Glycolipid Treatment Activates Tumor-Specific CD8+ T Cells and Overcomes Regulatory T Cell Activity

Adoptive transfer studies were performed with spleen lymphocytes from tumor-bearing mice treated with α-gal glycolipids that were transferred into naïve KO mice. These studies aimed to identify the lymphocytes that mediate the protective immune response against the tumor. The recipients were inoculated subcutaneously with 5×10^5 B16 cells. This inoculation was performed 24 h prior to the adoptive transfer of 40×10^6 spleen lymphocytes from B16 tumor-bearing donors treated with α-gal glycolipids or with PBS. In naïve mice that did not receive transferred lymphocytes, the challenging tumor cells developed into 5–7 mm lesions within 10 days and into a 20 mm lesion within 20–25 days. Adoptive transfer of lymphocytes from tumor-bearing mice treated with α-gal glycolipids resulted in prevention of tumor growth in ~70% of the recipients and the remaining recipients displayed slower tumor growth than in control mice [65]. However, when the transferred lymphocytes were depleted *in vitro* of CD8+ T cells (by anti-CD8-coated magnetic microbeads), the protective effect of the transferred lymphocytes was eliminated [65].

Lymphocytes transferred from mice with PBS-treated tumors had almost no protective effect and tumor growth was observed in >75% of the recipients [65]. Nevertheless, depletion of CD4+ T cells from the transferred lymphocytes resulted in increased protection against the tumor challenge [65]. These findings suggest that, in accordance with previous reports [66, 67], mice bearing B16 melanoma or other tumors have CD4+ regulatory T (Treg) cells that inhibit the development of a protective antitumor immune response. Thus, treatment with α-gal glycolipids seems to elicit a protective immune response potent enough to overcome the suppressive effect of endogenous Treg in the tumor-bearing mice [65].

10. Feasibility of α-Gal Glycolipid in Immunotherapy of Cancer Patients

In studies in humans, anti-Gal from human serum was found to induce effective targeting of tumor cells presenting α-gal epitopes for uptake by human macrophages and dendritic cells via Fc/Fcγ R interaction [68]. The *in vivo* safety of such targeting by α-gal glycolipid immunotherapy has been evaluated in a Phase I clinical trial under IND-12946 at UMass Medical Center in patients with advanced solid tumors. Patients with a variety of advanced cancers received a single intratumoral injection of α-gal glycolipid into one of their tumor lesions. The administration of α-gal glycolipids was performed by endoscopy, laparoscopy, or ultrasound guidance, depending on the site of the tumor. Using standard Phase I dose escalation, each cohort of patients received an intratumoral injection of 0.1, 1.0, or 10 mg α-gal glycolipids. All participating patients displayed no treatment-associated toxicity (manuscript in preparation). Based on these studies, the dose of α-gal glycolipids to be used in future Phase II studies is planned to be 10 mg. It is expected that the results of α-gal glycolipid treatment will vary from one patient to the other, depending on the immunogenicity of the various TAA in the individual patient and on the potency of the immune system in the treated patient. It is possible that in a proportion of the patients, the combination of effective TAA and potent immune system may result in the generation of a protective immune response against the autologous TAA that is effective enough to destroy tumor cells presenting these TAA.

The studies in mice strongly suggest that the elicited immune response against autologous TAA is potent enough

to destroy small groups of tumor cells, that is, micrometastases. However, it is not clear at present whether the α-gal glycolipid immunotherapy can elicit an immune response capable of destroying visible metastases. Studies in mice with B16 melanoma lesions [52] have demonstrated the effective destruction of the outer regions of visible tumors whereas the inner regions are not affected because of the inability of the T cells to infiltrate the core of such metastases. In view of the recent advances in immunotherapy by nonspecific modulators of the immune system such as the monoclonal Ab ipilimumab (anti-CTLA4 Ab enhancing T cell response), it may be possible that combination of such treatments with α-gal glycolipid treatment will have a synergistic immunoprotective effect. This is since the generation of activated T cells with autologous TAA specificity following the α-gal glycolipid treatment will be greatly enhanced by the subsequent "nonspecific" activation of the immune system by immunomodulators. Thus, it is possible that such a combination may induce a protective antitumor immune response that effectively destroys both micrometastases and visible metastases.

In addition to the possible use of α-gal glycolipids in immunotherapy of patients with advanced tumor, this treatment may be beneficial in improving prognosis when used as part of neoadjuvant immunotherapy in high-risk patients, prior to the resection of the primary tumor. One specific example may be mammary carcinoma. In a substantial proportion of women with mammary carcinoma, tumors originating from micrometastases reappear months to years after the resection of the primary tumor. In the suggested therapy, the primary mammary tumor is injected with α-gal glycolipids immediately after detection. The tumor is resected 3-4 weeks after this injection, as part of the standard clinical care. This time frame will provide a sufficient period for the APC to internalize autologous TAA, transport them to the draining lymph nodes, and activate the immune system to react against tumor cells expressing these TAA. Thus, long after the resection of the primary tumor, the immune system may be able to continue detecting and destroying tumor cells within micrometastases, thereby preventing the development of lethal metastases.

11. Conclusions

A protective immune response against tumors can be achieved by activating the immune system to react against the full range of autologous tumor-associated antigens (TAAs). Many of these TAA differ from one cancer patient to the other and are formed by various mutations due to genomic instability. Presently, it is difficult to identify the multiple autologous TAA in each patient in order to synthesize the various TAA peptides for vaccine preparation. Therefore, the tumor itself may serve as a source for the vaccinating TAA. In order for the tumor to function as a vaccine, tumor cells and cell membranes have to be effectively targeted to antigen-presenting cells (APCs) which process the TAA, transport them to the draining lymph nodes, and present the immunogenic TAA peptides for the activation of tumor-specific T cells. Tumor cells usually evolve to be "ignored" by APC and to develop without eliciting an antitumor immune response. Immunogenicity of tumors can be increased by manipulating them to express α-gal epitopes (Galα1-3Galβ1-4GlcNAc-R). Injection of glycolipids with α-gal epitopes (α-gal glycolipids) in the form of micelles into tumors results in spontaneous insertion of the lipid tail of these glycolipids into the cell membrane and the presentation of multiple α-gal epitopes on the tumor cell membranes. This results in binding of the natural anti-Gal antibody (constituting 1% of immunoglobulins in humans) to its ligand, the α-gal epitope on tumor cells within the treated lesion. Anti-Gal opsonizes the tumor cells and targets them to APC via the interaction between the Fc portion of the bound anti-Gal and Fcγ receptors on APC. Such interaction induces effective uptake of the opsonized tumor cells by APC, and subsequent processing and presentation of TAA peptides. The elicited immune response is potent enough to overcome the immunosuppressive effect of regulatory T cells and to activate tumor-specific T cells which can destroy tumor cells within micrometastases. A phase I study (IND 12946) in patients with advanced solid tumor has indicated that intratumoral injection of 0.1, 1.0, and 10 mg α-gal glycolipids has no adverse effects. This immunotherapy aims to destroy micrometastases in cancer patients with advance disease. In addition, injection of α-gal glycolipids into primary tumors few weeks prior to resection may convert the lesion into a temporary autologous tumor vaccine which induces a protective immune response that will destroy micrometastases, long after the primary tumor has been resected.

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Review Article

The Confluence of Stereotactic Ablative Radiotherapy and Tumor Immunology

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Stereotactic radiation approaches are gaining more popularity for the treatment of intracranial as well as extracranial tumors in organs such as the liver and lung. Technology, rather than biology, is driving the rapid adoption of stereotactic body radiation therapy (SBRT), also known as stereotactic ablative radiotherapy (SABR), in the clinic due to advances in precise positioning and targeting. Dramatic improvements in tumor control have been demonstrated; however, our knowledge of normal tissue biology response mechanisms to large fraction sizes is lacking. Herein, we will discuss how SABR can induce cellular expression of MHC I, adhesion molecules, costimulatory molecules, heat shock proteins, inflammatory mediators, immunomodulatory cytokines, and death receptors to enhance antitumor immune responses.

1. Introduction

Stereotactic radiosurgery (SRS) was originally developed for the treatment of intracranial tumors and has demonstrated clinical effectiveness in treating a variety of benign and malignant conditions. Its extracranial counterpart, stereotactic body radiation therapy (SBRT), also known as stereotactic ablative radiotherapy (SABR), has more recently shown efficacy for the treatment of tumors in organs such as the liver and lung. The potential for using SABR is likely greater than for SRS given the larger volume of potential indications outside the central nervous system. Technology, rather than biology, is driving the rapid adoption of SABR in the clinic due to advances in precise positioning, motion control, dosimetry, and precise targeting with image guidance. Dramatic improvements in tumor control have been demonstrated in several studies due to the demonstration that very potent dose can be delivered by use of the mentioned technology. However, our knowledge of normal tissue biology response mechanisms to large fraction sizes is relatively lacking compared to conventional fractionation.

2. Radiobiologic Considerations

A fundamental issue in SABR is whether classical radiobiologic modeling with the linear-quadratic (LQ) model is a valid method to assess the biologically effective dose at the high doses typically encountered in radiosurgery. This point was debated in back-to-back papers in seminars in radiation oncology [1, 2], where Brenner argued that LQ formalism was appropriate whilst Kirkpatrick and colleagues suggested it was inappropriate. Brenner's argument is based on the robustness of the LQ model to predict fractionation and dose-rate effects in experimental models *in vitro* and *in vivo* at doses up to 10 Gy. This conclusion is based on the premise that cell killing is the dominant process mediating the radiotherapeutic response for both early and late effects including vascular effects. Brenner argued that, to date, there

is no evidence of problems when LQ has been applied in the clinic.

However, this was the crux of Kirkpatrick and colleagues' argument. They noted multiple studies demonstrating that the administration of a single high dose of radiation *in vivo* had a much greater effect than predicted by the LQ model; they cited several examples including Leith et al. [3] who calculated that the dose to obtain a high probability of tumor control for brain lesions would be at least 25 to 35 Gy using the LQ model, which was much higher than the observed clinically effective radiosurgical dose, which was in the range of 15–20 Gy. Kirkpatrick maintained that there was a disconnect between *in vitro* cell survival data and observed clinical data which suggests that there is more than one mechanism of radiation damage and that these operate differentially at low and high doses. In addition, Kirkpatrick argues that the LQ model does not effectively address the potential existence of radioresistant cancer stem cells, which may require a threshold dose to be crossed before their death is triggered.

Several authors have proposed alternate models to the LQ. In all cases, they argue that the LQ model was intended as a low dose mathematical representation of the data constituting the survival curve [4, 5]. As most survival curves demonstrate a curvilinear "shoulder" followed by a linear portion on a linear log scale, the high-dose trend to endlessly curve associated with the LQ model overpredicts survival at high dose per fraction from a purely mathematical perspective. In the case of the universal survival curve of Park et al. [5], the strength of the LQ in the low dose realm is exploited but abandoned for the linear multitarget model in the high-dose realm. Thus, the *in vitro* survival curve has goodness of fit in all clinically significant ranges including the ablative range characteristic of SABR. Admittedly, none of the proposed mathematical models properly account for *in vivo* effects including vascular and immune contribution to cell death.

3. The Role of Tumor Stroma

As stated above, the accepted rationale for radiotherapy (RT) is based on causing lethal DNA damage to tumor cells and the tumor-associated stroma. There is unequivocal evidence which has been presented by Fuks and colleagues that the tumor stroma plays an important role in the response to high dose per fraction radiation treatment. They demonstrated that vascular endothelial cell apoptosis is rapidly activated above 10 Gy per fraction [6], and that the ceramide pathway orchestrated by acid sphingomyelinase (ASMase) operates as a rheostat that regulates the balance between endothelial survival and death and thus tumor response [7]. These studies relied heavily on mice that had ASMase knocked out in all tissues; the authors have countered the argument that defective immune system that is known to occur in ASMase −/− mice [8] influenced their observations [6].

Damage to vascular/stromal elements in tumors has also been observed around 2 weeks after radiation exposure that was less dependent on size of dose per fraction [9]. Pathological observations show profound changes in vasculature after radiosurgery and from studies on arteriovenous malformations [10], where obliteration of abnormal vasculature occurs months after irradiation, but is rarely seen below single doses of 12 Gy climbing steeply with increasing doses above this threshold.

In terms of the infiltrating immune cell component of tumor stroma, conventional RT has traditionally been viewed as immunosuppressive [11], but the systemic effects of both cancer and local radiotherapy of cancer on the immune system are clearly more complex than this. Although lymphocyte radiosensitivity is well recognized, the effects of different doses and delivery methods on systemic and locoregional naive, effector, or regulatory T cell or other immunologically relevant populations is still the subject of debate [12, 13]. Several authors have investigated the potential immunomodulatory effects of localized RT on tumors resulting in conflicting reports as to whether these responses promote or interfere with tumor reduction [14–16]. This dualism is something that is to be expected and is inherent in a system that has to promote both destruction of pathogens and tissue healing while regulating anti-self reactivity. It is also possible that the more positive effects seen in colorectal cancer where the immune score was significantly associated with differences in disease-free, disease-specific, and overall survival [17] are in part a reflection of additional microbial challenges that may not be present in other sites.

4. Direct Interaction between Radiation, Tumor Cells, and the Immune System

Several lines of evidence have suggested direct immune modulation of immune cells by RT [15, 18–22]. Apetoh et al. showed that radiation can trigger signals that stimulate toll-like receptor 4 on antigen presenting dendritic cells (DCs) [18], Liao has shown that irradiation of DC can enhance presentation of antigenic peptides by the exogenous pathway and is a maturation signal, while inhibiting internal antigen processing [21], and Merrick has shown a decrease in IL-12 production that has a negative effect on presentation [15]. Several reports have shown increased expression of MHC class I and coaccessory molecules after radiation of both tumor and host cells, while Chakraborty et al. [19] reported a direct effect of radiation on tumors by modifying the phenotype of tumor cells to render them more susceptible to vaccine-mediated T-cell killing, and others have shown that radiation-induced changes in the tumor immune microenvironment to promotes greater infiltration of immune effector cells [22] (Figure 1).

5. Mechanisms of Radiation Driven Tumor Immunology

The early report of Stone [23] that the immune system can dramatically alter the dose required to obtain local tumor control has been updated by Lee and colleagues, who showed that CD8+ T cells could be responsible for the therapeutic effects of ablative radiation [24]. The delivery of an ablative dose of radiation of 15–25 Gy was found to cause a significant increase in T-cell priming in draining lymphoid tissue, leading to reduction or eradication of the primary tumor or distant metastasis in a CD8+ T-cell dependent fashion in an

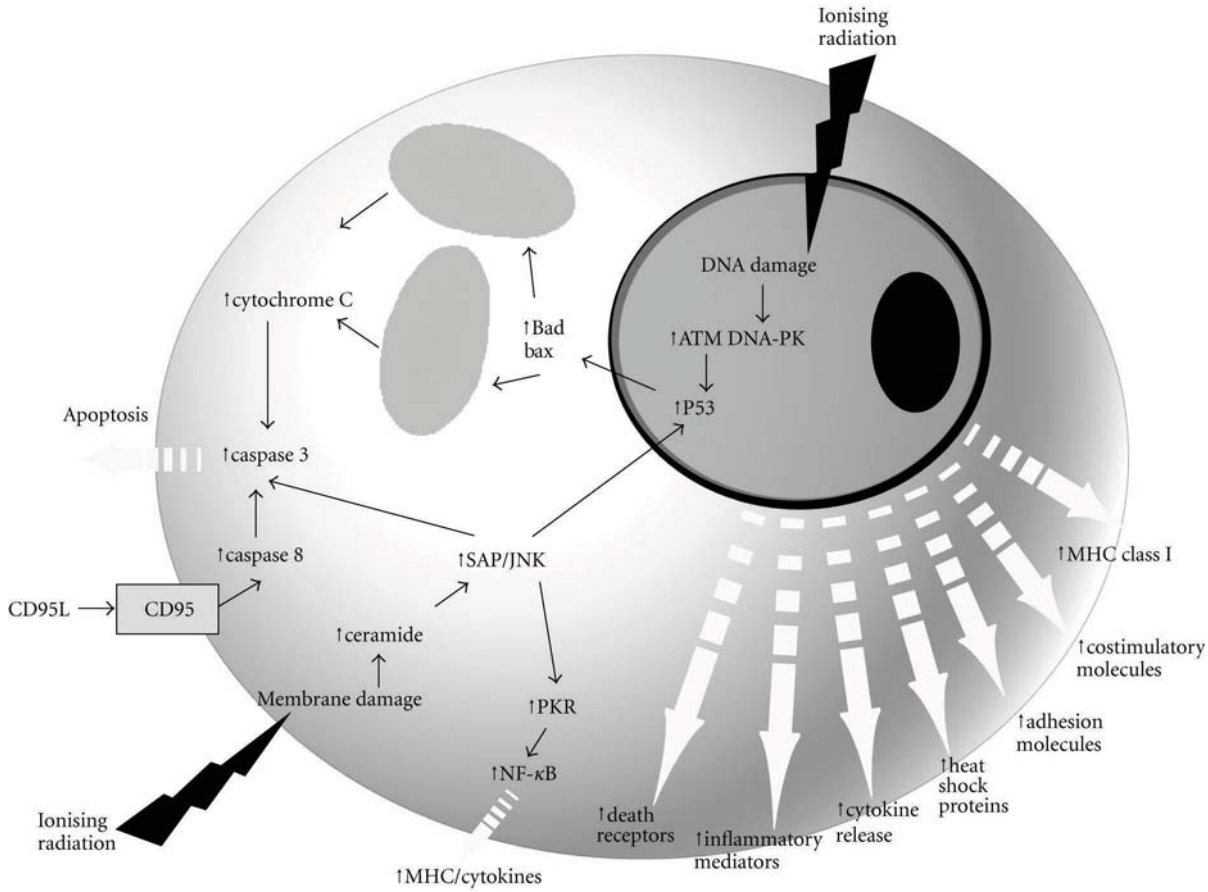

Figure 1: *Confluence of SABR and Immunotherapy.* Apoptosis can be initiated by SABR-induced DNA damage and upregulation of the p53 tumor suppressor gene. In addition, apoptosis can be triggered by SABR-induced damage to the cellular lipid membrane, which can induce ceramide formation and activate the SAPK/JNK signaling pathway. Thus, SAPK/JNK can upregulate PKR expression, which can induce MHC and cytokines via NF-κB. SABR can induce cellular expression of MHC Class I, adhesion molecules, costimulatory molecules, heat shock proteins, inflammatory mediators, immunomodulatory cytokines, and death receptors.

animal model. While conventional 2 Gy doses seem inferior at generating such responses, higher sized dose fractions may be better than single doses [25].

The possibility that there may be a certain dose per fraction that is optimal for stimulating radiation adjuvanticity is of relevance to mechanism of radiation-induced immune stimulation and clinical practice. Conventional RT has already been shown to enhance tumor-specific T-cell responses [26], but such responses are likely of little clinical relevance and surely can be improved upon by optimizing dose delivery and integrating RT with modern immunotherapeutic strategies.

Radiation can not only kill tumor cells releasing tumor antigens and molecules with what are collectively called damage-associated molecular patterns (DAMPs) that exert various immunomodulatory effects including induction of the expression of cytokines, chemokines, and release of inflammatory mediators [27–30] (Figure 1). Although pro-inflammatory cytokines generally are produced by higher doses than are conventionally used in RT, there may be an accumulating effect [31]. Radiation also increases the permeability of the local vasculature either directly or through cytokine production that leads to recruitment of circulating leukocytes into surrounding tissues including antigen-presenting cells and effector T cells [32–34]. Thus, a radiation-induced proinflammatory microenvironment within irradiated tumors could provide DCs with maturation inducing stimuli critical for eliciting effective antigen presentation. The obverse of this is that radiation can stimulate production of suppressor myeloid cells [35] and Treg cells [36] in a dose-dependent manner that presumably aim to dampen and contain tissue damage and that can be highly immunosuppressive. Thus, to "unmask" the more positive aspects of radiation killing on immunity, it may be necessary to target and impair these natural defenses.

Advances in the understanding of the mechanisms that regulate the development of antitumor immunity, as well as improved knowledge of the complex effects of radiation on tissues [37], have revived interest in the possibility of combining radiation and immune-based therapies to achieve a better local and systemic tumor control [28–31]. Since William Coley started treating patients at the end of the 19th

century with bacterial toxins, there have been waves of enthusiasm promoting immunotherapy for the treatment of cancer. The introduction of cytokines, in particular interleukin-2 (IL-2), for cancer treatment was a major clinical effort that had modest success. Until recently, however, these efforts have been hampered by a lack of molecular definition of tumor antigens, a means of delivering them effectively, and a sensitive and reliable way to measure responses.

This situation changed with the molecular cloning of human tumor-associated antigens that could be recognized by T cells, the ability to culture powerful antigen presenting cells (APCs) in the form of dendritic cells (DCs), and to assess immune responses to specific tumor epitopes using tetramer and ELISPOT assays [38]. These advances allied to the development of genetically modified mouse models have led to a deeper understanding of the interactions between cancer and the immune system of the host [39]. Indeed, the available experimental evidence supports the hypothesis that once tumors have become clinically apparent their immunogenicity has been modified by the selective pressure of the immune system, resulting in the growth of tumors that are characteristically poorly immunogenic, being able to escape immune detection, and/or to actively inhibit immune effectors [39]. Furthermore, it is clear that, although T cells become tolerant to many self-antigens in the thymus, which depletes the pool that might react to cancer, tolerance to many self-components is actively maintained in the periphery by several mechanisms. For example, immature DC presenting self-antigens to T cells are tolerogenic and peripheral tolerance is maintained by Tregs subset that can be innate or induced. Suppressor macrophages form a final barrier to immune function and can result in immune shutdown [40]. Peripheral tolerance can be broken by "maturation" of DC in local sites that allow transient immune responses to invading pathogens, but it leads to the belief that if it were not for these regulatory mechanisms T cells could respond better to "self-" antigens on tumors, something for which there is now considerable evidence [41].

The recognition of the fact that the host can break a state of tolerance that has developed to its own tumor offers many possibly effective immunotherapeutic strategies, some being currently tested in clinical trials. The "danger" model of immunity suggests that pathogens with associated molecular patterns (PAMPs) and DAMPS engender an inflammatory milieu that promotes the development of antigen-specific immunity through DC maturation that allows internalization of apoptotic and necrotic cellular debris and presentation of processed antigen to T cells. Thus, administration of radiation may therefore be considered to create an inflammatory setting via DC maturation, induction of apoptosis, necrosis, cell surface molecules, and secretory molecules. As with many other challenges, radiation upregulates expression of immunomodulatory surface molecules (MHC, costimulatory molecules, adhesion molecules, death receptors, heat shock proteins) and secretory molecules (cytokines, inflammatory mediators) in tumor, stromal, and vascular endothelial cells. Important amongst these may be the upregulation of TNF family members that could promote cell killing, not only by TNF in the microenvironment but also by radiation-induced TNF.

6. Can Radiobiologic Models Be Adapted to Account for Other Modes of Tumor Response at High Dose Per Fraction?

Therefore, the evidence would seem to suggest that there are several potential immunologic mechanisms for cell killing in the high-dose range. The LQ model has long been considered to overestimate radiation cell killing at these doses as a consequence of the model's prediction of a continuous downward bend (ßd2) in the survival curve. While *in vivo* data are sparse, the dose-response may be linear above 12 Gy [42], and two-component or other models have been described that may better predict the response at dose per fraction above 5–7 Gy. For example, Park et al. [5] described the effects of radiation in the ablative dose range using a universal survival curve (USC) model, which combines the LQ and multitarget models using a transition dose to separate the two fitting components of the model. Using the LQ model, the potency of the doses used in the Indiana University phase II trial of SABR for medically inoperable NSCLC (20 Gy $\times$ 3) was estimated to be 1.7 times greater than the biological effectiveness of a similar Japanese trial delivering 12 Gy $\times$ 4. However, when the USC model was used, the potency of the Indiana University regimen was only 1.34 times more than the Japanese regimen [5]. Other models have included the generalized LQ (gLQ) model in which the reduction of conversion of sublethal to lethal injury in hypofractionated ablative dose radiation is taken into account and the actual effect of the radiation is lower than what was estimated by the LQ model [43]. However, modeling may never fully describe the complexity of the biological processes involved in the response to high dose per fraction radiation, but it might facilitate the ability to design optimal radiosurgery treatment plans once sufficient clinical data have been obtained. From a radiobiological perspective, what is clear is that there are processes that are different at high from low dose per fraction and these include the ability of cells to progress through the cell cycle, the likelihood of cell death perhaps with a different mechanism, vascular effects, proinflammatory effects, and immune effects.

7. Local Radiation Enhancement of Systemic Immunity

It is clear from what has been said that localized cancer has systemic immune effects as does RT. It is also clear that the outcome of cancer and cancer therapy depends heavily upon the nature of the cells that are generated, in particular with respect to metastasis and overall survival. It seems likely that unexpected discrepancies in the relative efficacies of different anticancer regimens and divergence or convergence between regional and distant failures could be due to such systemic influences, for example, of local tumor control on the incidence of distance metastasis. Future studies aimed at assessing the predictive value of systemic responses in the response of cancer to different dose schedules of RT

are likely to be very informative, and strategies that target systemic innate and cancer and radiation-induced regulatory mechanism hold great promise. These strategies, together with DC-based and other forms of antitumor vaccination, can greatly modify the total radiation dose required to achieve local control as well as influencing distant disease, and RT should adapt to optimally integrate with such approaches. While most chemotherapy regimens are thought to compromise the immune system, they also can have immunomodulatory effects that require study.

8. Conclusions

Searching for references on PubMed that contain "SBRT" or "SABR" and "biology" reveals very few hits emphasizing that this is an area of modern radiotherapy where detailed understanding biology needs to catch up with the clinic [44]. Small animal platforms are now developed to simulate a realistic SABR delivery in experimental animals [45] and other recent developments in image-guided small animal irradiators could also be adapted to simulate SABR [46]. A wealth of knowledge already exists in the radiobiology archive from the '60s, '70s, and '80s where large doses per fraction were used for ease of experimental design in experimental studies, which needs to be revisited. In the meantime, combination immunotherapy and radiation approaches are being translated into the clinic [47]. Currently, combination immunotherapy and radiation approaches are being translated into the clinic where intratumoral dendritic cell injection with coordinated irradiation and introduction of autologous, unmanipulated dendritic cells has been the subject of sarcoma therapy [48].

At present, SABR represents an exciting, effective, yet empirically designed radiation therapy. Increasing our knowledge of the underlying biology associated with modern high-dose delivery will only serve to improve the therapeutic benefit of this modality. In addition, we believe that SABR could be optimized for use with immunotherapeutic approaches so as to better generate tumor antigen-specific cellular immunity.

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Clinical Study

Patients with Multiple Myeloma Develop SOX2-Specific Autoantibodies after Allogeneic Stem Cell Transplantation

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The occurrence of SOX2-specific autoantibodies seems to be associated with an improved prognosis in patients with monoclonal gammopathy of undetermined significance (MGUS). However, it is unclear if SOX2-specific antibodies also develop in established multiple myeloma (MM). Screening 1094 peripheral blood (PB) sera from 196 MM patients and 100 PB sera from healthy donors, we detected SOX2-specific autoantibodies in 7.7% and 2.0% of patients and donors, respectively. We identified $SOX2_{211--230}$ as an immunodominant antibody-epitope within the full protein sequence. SOX2 antigen was expressed in most healthy tissues and its expression did not correlate with the number of BM-resident plasma cells. Accordingly, anti-SOX2 immunity was not related to SOX2 expression levels or tumor burden in the patients' BM. The only clinical factor predicting the development of anti-SOX2 immunity was application of allogeneic stem cell transplantation (alloSCT). Anti-SOX2 antibodies occurred more frequently in patients who had received alloSCT ($n = 74$). Moreover, most SOX2-seropositive patients had only developed antibodies after alloSCT. This finding indicates that alloSCT is able to break tolerance towards this commonly expressed antigen. The questions whether SOX2-specific autoantibodies merely represent an epiphenomenon, are related to graft-versus-host effects or participate in the immune control of myeloma needs to be answered in prospective studies.

1. Introduction

SRY-related HMG box (SOX) is a superfamily of transcription factors involved in embryonic development and stem cell function [1]. Cancer cells share pathways regulating pluripotency with embryonal stem cells [2], and some of the transcription factors involved, including SOX2, have indeed been identified as lineage survival oncogenes in epithelial cancers [3]. The impact of SOX2-specific immunity on the patient's prognosis has been investigated in single solid tumors [2]. However, the exact biological role of cancer-related SOX2-specific antibody and/or T cell responses has remained unclear. Accordingly, some studies have suggested an association with an improved prognosis while others have found no association with the patients' outcome or have even described a negative impact on the course of the disease [4–6].

Monoclonal gammopathy of undetermined significance (MGUS) is a premalignancy converting to symptomatic multiple myeloma (MM) at a rate of 1-2% of patients per

year [7]. The prevalence of SOX2-specific antibodies in MGUS patients has been linked to a decreased risk of progression to MM [8]. However, SOX2 is expressed not only in MGUS but also in symptomatic MM [6], and it has remained unclear if and under which clinical conditions autoantibodies against SOX2 also occur in established MM. Moreover, allogeneic stem cell transplantation (alloSCT) has been suggested to break tolerance towards different tumor antigens in MM resulting in a clinically relevant graft-versus-myeloma (GvM) effect. The question is still open, however, if alloSCT also influences the development of anti-SOX2 immunity in patients with established MM [9, 10]. To address these issues, we performed a longitudinal analysis of SOX-specific antibodies in patients with established MM.

2. Material and Methods

2.1. Patients. Patients were admitted for diagnostic purposes and/or treatment to the University Medical Center Hamburg-Eppendorf. Repeated blood samples were obtained during routine diagnostic procedures and all participants provided informed consent prior to sample collection. A total of 1094 peripheral blood (PB) plasma samples and 25 bone marrow (BM) samples were collected from 194 consecutive MM patients. In addition, 100 PB sera and 10 BM samples were collected from healthy donors. Samples were collected as previously described [11]. This study was conducted in accordance with the declaration of Helsinki. The protocol had received approval by the local ethics committee (decision number OB-038/06).

2.2. Myeloma Cell Lines. Cell lines U266, RPMI 8266, LP1, OPM2, NCIH929, MOPL8, KMS12BM, IM9, and EJM were obtained from the German Collection of Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany). Cell line SK 007 was provided by the Ludwig Institute for Cancer Research (LICR), New York branch. Cell lines were maintained in RPMI 1640 and 10% fetal calf serum [12].

2.3. Proteins and Peptides. Full-length SOX2 protein and control protein glutathione *S*-transferase (GST) were expressed in a wheat germ system (Abnova, Taipei, Taiwan). Recombinant influenza nucleoprotein (FLU) produced in *E. coli* was obtained from Imgenex (San Diego, Calif) and tetanus toxoid (TT) was provided by Chiron Behring (Marburg, Germany). Control protein for FLU and TT antibody detection was GST expressed in *E. coli* (Cell Systems, St Katharinen, Germany). 20 mer SOX2 peptides ($n = 31$) spanning the entire SOX2 sequence consisting of 317 amino acids were obtained from Iris Biotech (Marktredwitz, Germany).

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). 96-well plates were coated over night at 4°C with recombinant protein or peptides diluted in PBS at a final concentration of 1 μg/mL, if not otherwise specified. Plates were blocked with PBS containing 3% milk powder for two hours at room temperature (RT). Sera were diluted 1 : 100 in 5% milk powder in PBS (MPBS) and incubated for two hours at RT. A secondary alkaline phosphatase-conjugated anti-human IgG antibody (Southern Biotech, Birmingham, Ala) diluted 1 : 3000 in MPBS was applied for one hour at RT. Detection reagent para-nitrophenylphosphate (PNPP; Southern Biotech) was added to the plates, and the phosphatase reaction took place at RT for 30 minutes, before reaction arrest with 3 N NaOH. Specific absorption was measured at 405 nm using a Sunrise ELISA reader (Tecan, Crailsheim, Germany).

In the screening part of the study, a sample was considered antibody positive if the OD measured was higher than the mean OD of 100 samples from healthy donors + 3 SD. In addition, the OD was required to exceed the autologous background signal measured with control protein GST by at least 50%. In the titration part of the study, serial serum dilutions were performed for antibody-positive samples, and results obtained with GST protein were used as reference values. For calculation of titers, regression analyses were performed for the linear segment of the serum titration curves for the patient sample and pooled sera of five representative healthy donors. Titers were defined mathematically as the dilution at the intersection of both regression lines.

2.5. Real-Time PCR. Extraction of total RNA was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription and quantitative PCR were performed as previously described [10]. The primer sequences for SOX2 were as follows: forward 5′-GCA CAT GAA CGG CTG GAG CAA CG-3′, reverse 5′-TGC TGC GAG TAG GAC ATG CTG TAG-3′. Samples were analyzed using a LightCycler system (Roche Diagnostics, Risch, Switzerland), and relative quantification was carried out by normalization against GAPDH RNA.

2.6. Western Blot. Protein lysates were prepared using standard lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich, Hamburg, Germany) and were subsequently denaturated for 10 min at 70°C. Samples of lysates or recombinant protein containing 500 μg and 300 μg of total protein, respectively, were separated using 4–12% Bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, Calif) under reducing conditions. Proteins were blotted on Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ), blocked overnight at 4°C with Top-Block (Fluka, Buchs, Switzerland). Human sera were applied at a dilution ranging between 1 : 500 and 1 : 2000. An HRP-conjugated anti-human IgG-Fcγ antibody (Sigma Aldrich) was used as secondary antibody at a dilution of 1 : 5000. β-Actin (Santa Cruz) served as loading control.

2.7. Flow Cytometry. For the analysis of cytoplasmatic SOX2 protein expression, myeloma cell lines or bone marrow mononuclear cells were first stained using a CD138-FITC monoclonal antibody (clone B-A38, BD Biosciences). Next, cells were fixed using FACS Lysing Solution (BD Biosciences)

and were permeabilized using Permeabilizing Solution (BD Biosciences). Cytoplasmic staining was performed applying a PE-conjugated SOX2 antibody (clone IC2018P, R&D, Abington, England) or an appropriate isotype control. Samples were analyzed using a FACSCalibur cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, Ore).

2.8. Epitope Prediction. Web-based prediction of potential SOX2 antibody epitopes (http://www.cbs.dtu.dk/services/BepiPred) was performed using the method published by Larsen et al. [13].

2.9. Statistical Analysis. Statistical analyses were performed using GraphPad software. The Mann-Whitney U test was used to calculate differences between different patient cohorts. Analysis of covariance was used to assess correlations between plasma cell count, SOX2 antibody titers, and SOX2 expression. Correlations between clinicopathological variables and occurrence of SOX2 antibodies were done by Pearson's χ^2 test. Differences were regarded significant if $P < 0.05$.

3. Results

3.1. SOX2 Is Expressed in Various Healthy Tissues and Malig nant Myeloma Cells. SOX2 has been reported to be overexpressed in malignancies [14–16], and overexpression of SOX2 has been associated with immunity towards autologous antigens in cancer patients [17]. Therefore, we first addressed the expression of SOX2 in the BM of MM patients compared to other tissues. To this end, we screened a wide variety of normal tissues including 10 BM samples from healthy donors as well as BM samples from 25 MM patients for SOX2 expression by real-time PCR (Figure 1(a)).

We found SOX2 RNA to be ubiquitously expressed in all tissues analyzed. SOX2 is an intronless gene, and, therefore, this expression could also represent an artifact due to the presence of genomic DNA within the samples. However, we could rule out this possibility by showing that no SOX2 expression was detectable when the PCR was performed with non-reverse-transcribed RNA samples (Figure 1(a)). Importantly, we did not detect any significant differences in BM expression of SOX2 between myeloma patients and healthy donors (Figure 1(a)). To prove the presence of SOX2 on the protein level, we have performed flow cytometry analysis of myeloma cell lines and of plasma cells of healthy donors, all of which we found positive for SOX2 by RT-PCR. Importantly, all of the myeloma cell lines, all peripheral, and one of two bone marrow-derived plasma cell samples were also found positive for SOX2 on the protein level (Figure 1(b)). These data demonstrate a strong correlation between expression on the RNA level and protein expression as indicated by flow cytometry. As suggested by the comparable expression of SOX2 in normal and malignant plasma cells and its broad expression in different healthy tissues, copy numbers of SOX2 RNA as measured by quantitative PCR did not correlate with the percentage of myeloma cells within the BM of our MM patients (Figure 1(c)).

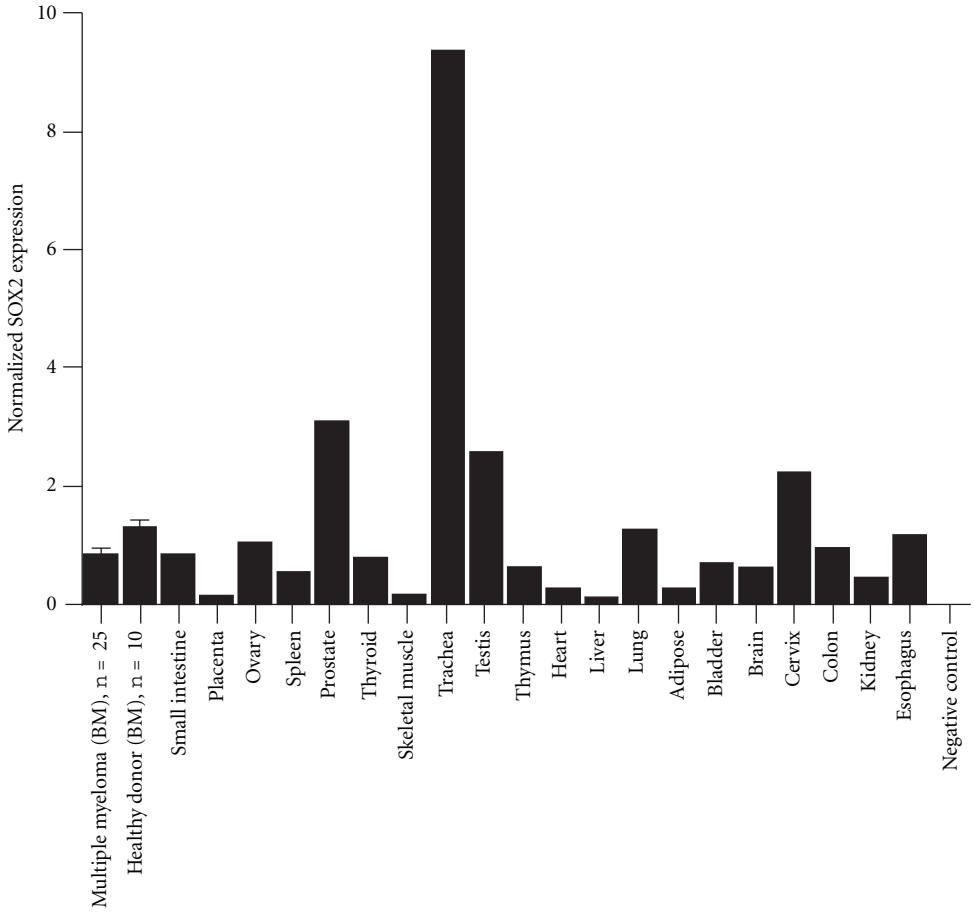
3.2. Antibodies against SOX2 Recognize the Natural Protein and Occur More Frequently in MM Patients Than in Healthy Donors. We next screened a large number of sera ($n = 1094$) consecutively collected from myeloma patients ($n = 196$) as well as sera from healthy blood donors ($n = 100$) for antibody responses against SOX2. Analyzing a median number of 5.4 (range 1–47) serum samples collected per patient during a median follow-up period of 11.4 months (range 1–39 months), we found 7.7% (15/196) of MM patients and 2% (2/100) of healthy donors to experience autoantibodies against SOX2 (Figures 2(a) and 2(b)). Out of all samples consecutively collected from our myeloma patients, 2% (68/1094) were positive for anti-SOX2 IgG antibodies. Overall, myeloma patients showed a significantly higher frequency of anti-SOX2 antibodies than healthy controls ($P < 0.05$, Figure 2(c)).

To address the general seroreactivity of the included patients and donors against common microbial and viral antigens, we screened all samples for the presence of antibodies against influenza virus nucleoprotein (FLU) and tetanus toxoid (TT). Importantly we did not detect any difference in the frequency of naturally occurring or vaccine-induced antibody responses between myeloma patients and healthy donors (Figure 2(b)). This result strongly suggests that frequency of anti-SOX2 immune responses in MM patients was not influenced by a general hypogammaglobulinemia or B-cell hyporeactivity.

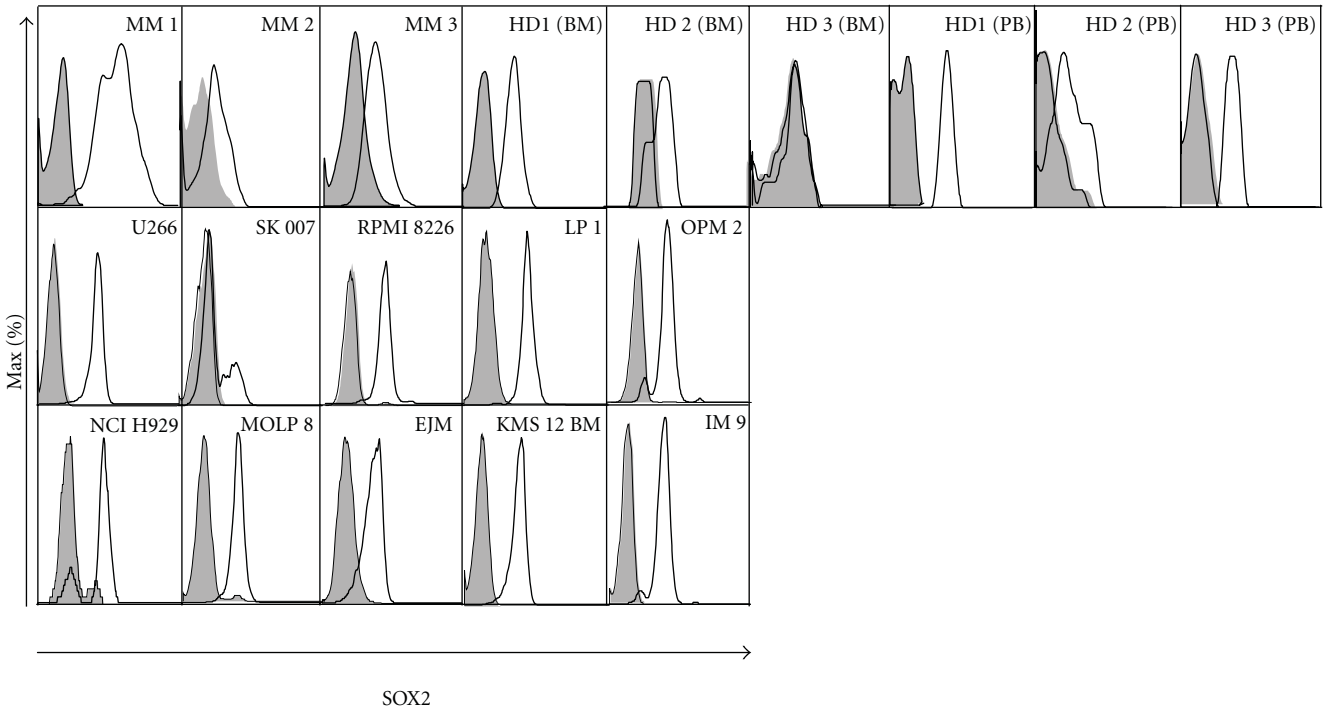
To confirm the specificity of our patients' serum antibodies, recognition of SOX2 was analyzed by western blot (Figure 3(a)). We found that the IgG antibodies in the patients' sera recognized both the recombinant SOX2 protein and SOX2 protein expressed by a myeloma cell line (U266) shown to be positive for SOX2 (Figure 3(a)). On the other hand, a control protein and a SOX2-negative tumor cell line (DLD-1) remained undetected by the patient serum.

3.3. $SOX2_{211-230}$ Represents an Immunodominant Epitope Recognized by Autoantibodies in Myeloma Patients. To further address the specific target of the anti-SOX2 antibody responses, we mapped epitopes recognized using 31 overlapping 20 mer peptides spanning the complete sequence of the antigen. In one-third (5/15) of the patients we were not able to detect any peptide-specific responses suggesting that the respective antibodies might recognize conformational epitopes. However, in the majority of the seropositive patients (53.3%) SOX2-specific antibodies targeted amino acid region 211–230 (Figure 3(b)). Other epitopes were much less frequently recognized by the patient-derived anti-SOX2 IgG antibodies. Using a hidden Markov prediction algorithm we predicted the potential epitopes of target of a SOX2 specific-antibody response (Figure 3(c)). Remarkably, the region with the highest score was indeed the region preferentially targeted by the majority of SOX2-specific antibody responses (211–230).

3.4. SOX2-Specific Autoantibodies Are Preferentially Induced after Allogeneic Stem Cell Transplantation. In order to understand which clinical factors might be associated with the development of anti-SOX2 antibodies in MM, we next cor-



(a)



(b)

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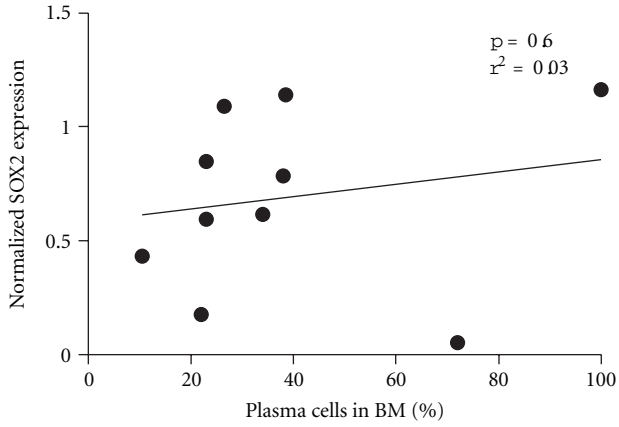

(c)

Figure 1: (a) RT-PCR analysis of SOX2 expression normalized to GAPDH in human tissues. BM from MM patients ($n = 25$), healthy donors ($n = 15$), myeloma cell lines ($n = 10$), and 20 human tissues ($n = 1$) was screened for SOX2 expression. Aqua dest. and non-reverse-transcribed mRNA were used as negative controls. 20 organs were tested for the presence of contaminating DNA. The resulting copy numbers (reverse-transcriptase-free) were normalized to GAPDH copy number of the respective tissue (cDNA). The mean value of all reverse-transcriptase-free results was calculated and included as the reverse-transcriptase-free (RT-free) condition. (b) FACS analysis of three MM patients' BM, three BM of healthy donors, and three peripheral blood samples of healthy donors for SOX2 expression in gated CD138+ plasma cells. One BM sample (3) was found negative for SO2 protein expression. SOX2 expression was also found in 10 different myeloma cell lines. Isotype antibodies served as negative control for SOX2 expression. (c) Correlation analysis of SOX2 expression and percentage of plasma cells in the BM of MM patients. No significant association between SOX2 expression and the amount of plasma cells was found ($P = 0.6018$, $r^2 = 0.03556$). HD: healthy donor; MM: multiple myeloma; BM: bone marrow; PB: peripheral blood.

related a number of clinicopathological attributes of our patients with the presence or absence of such serological responses. As expected from our observation of a missing association between SOX2 antigen expression and the number of BM-infiltrating plasma cells, we did not observe a correlation between the presence of anti-SOX2 antibodies and the tumor load in the respective patient (Figure 4(a)). Most of the remaining clinicopathological parameters also lacked an association with the appearance of a humoral response against SOX2 (Table 1).

Since myeloma treatment, particularly stem cell transplantation, has immune-modulating properties, we finally investigated the relationship between therapeutic interventions and the occurrence of SOX2-specific antibodies in the myeloma patients. Remarkably, 92.6% (63/68) of all samples found positive for anti-SOX2 antibodies were collected after the patient had received alloSCT. In contrast, only 4.4% (3/68), 0% (0/68), and 2.9% (2/68) of the SOX2 antibody-positive samples were derived from time points when the patient had been treatment naïve, had only received conventional chemotherapy, or had been treated with autologous stem cell transplantation (autoSCT) as maximum therapy (Figure 4(b)). Accordingly, 80.0% (12/15) of the anti-SOX2 antibody-positive MM patients had received alloSCT as maximum therapy while only 13.3% (2/15), 0% (0/15) and 6.6% (1/15), were treatment naïve or had been treated with conventional chemotherapy or autoSCT as maximal therapy, respectively (Figure 4(c)). Importantly, anti-SOX2 antibodies are not likely to be a marker of an unspecific graft-versus-host reaction, since none of the SOX2 antibody positive patients suffered from GvHD at any time point after alloSCT.

To further address the impact of alloSCT on SOX2-specific immunity, we screened samples from the alloSCT, patients taken before transplantation. From 10 available pre-alloSCT samples 9 were found to be negative for SOX2 antibodies prior to alloSCT and these patients had experienced a seroconversion at a median of 21.5 months (range 1–87 months) after transplantation (Figure 4(d)). This observation strongly suggests that immunological mechanisms induced by alloSCT may be capable of breaking tolerance towards SOX2.

To investigate if, as suggested before, SOX2 antibodies may protect from disease progression or recurrence [6], we correlated SOX2 antibodies with the clinical remission status of the patient. We found seropositive samples to be evenly distributed between patients with clinical remission (56%) or progressive disease (44%), respectively (see Supplementary Figure 1 in Supplementary Material available online at doi: 10.1155/2011/302145). This finding suggests that there is no immediate connection between the presence of anti-SOX2 humoral immunity and the clinical response of a given MM patient.

4. Discussion

We hereby report SOX2 to be expressed in all tissues we analyzed (Figure 1(a)), which is in contrast to previous studies where SOX2 expression has been reported to be restricted to certain tissues (among others neural, stem cell, or tumor tissue) [3, 18]. However, the EMBL-EBI database (http://www.ebi.ac.uk/gxa/gene?gid=P48431) which comprises a meta-analysis of all gene expression data available for SOX2

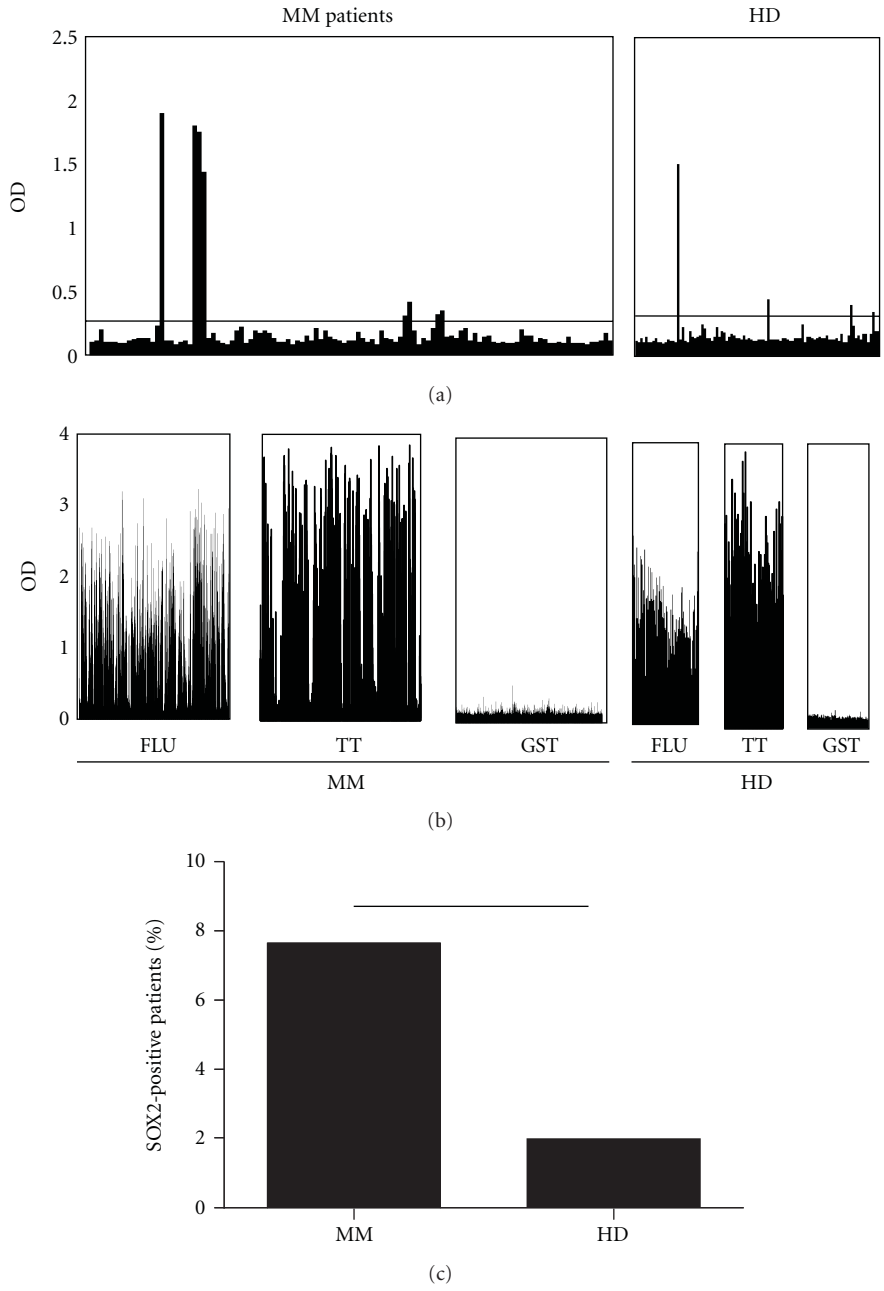

Figure 2: (a) Analysis of SOX2-specific IgG antibody responses in MM patients ($n = 1094$) and in healthy donors ($n = 100$). Results are shown as optical density (OD) at 405 nm. Horizontal bar represents the cut-off value for positivity (OD > 0.274). (b) Analysis of influenza-nucleoprotein- (FLU-), tetanus-toxoid- (TT-) and glutathione-S-transferase- (GST-) specific antibody responses in the same collective of MM patients and healthy donors. (c) Incidence of SOX2-specific antibody responses in the group of MM patients compared with the group of healthy donors (7.7% versus 2.0%). We found significantly more individuals with SOX2-specific antibody responses in the MM group than in the healthy donor group ($P < 0.05$).

reports expression in all parts of the human body and in many disease states. In particular, this database reports an expression of SOX2 in plasma cells and myeloma cells, as confirmed by our data (Figure 1). In our current study, SOX2 was not differentially expressed in the BM of healthy donors when compared to the BM of MM patients, an observation which would be in line with the latter analysis. While plasma cells in general might indeed express comparably high levels of SOX2 [19, 20], we believe that SOX2 is by no means tumor or myeloma specific. This assumption is further supported by our flow cytometry data (Figure 1(b)) which suggests a similar expression of SOX2 in the BM of MM patients, in MM cell lines, and in the BM and PB of healthy donors.

A single study has previously addressed SOX2-specific antibodies in plasma cell disorders [6]. Spisek and colleagues reported SOX2-specific antibodies in 23% of MGUS patients

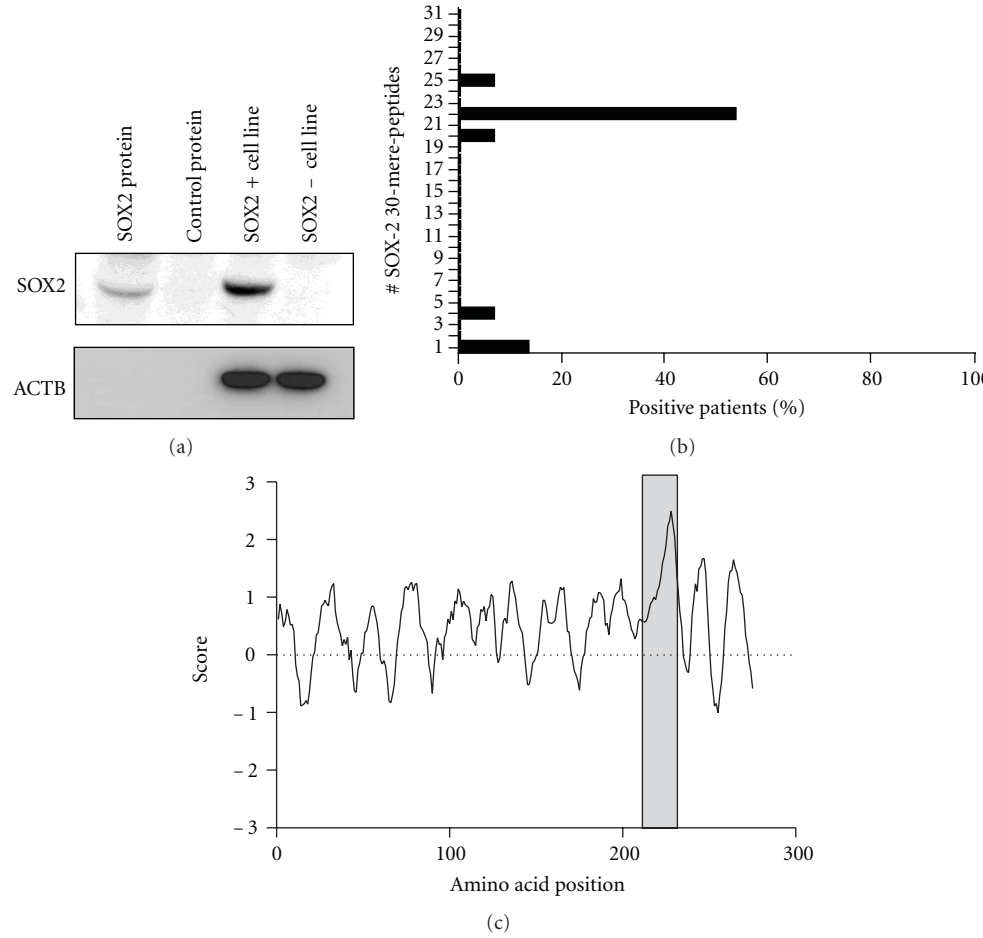

Figure 3: (a) Analysis of specificity of the SOX2 targeted IgG antibody response. Serum from a patient found positive by ELISA was used for western blot analysis and specifically recognized recombinant SOX2 and SOX2 from a SOX2-positive cell line (U266). In contrast, GST and a SOX2-negative cell line (DLD1) remained unstained. ACTB was used as loading control. (b) Mapping of the epitopes of target of the SOX2-specific antibody response in MM patients. Overlapping 20 mer peptides ($n = 31$) spanning the complete SOX2 sequence were used. Percentages of SOX2-antibody-positive patients for each epitope are given on the x-axis. Three patients recognized two or three epitopes. 8 patients had SOX2-specific antibodies only directed against the 20 mer 22 (amino acids 211–230), and the antibodies of five patients did not recognize any of the 20 mers that were used. (c) Epitope prediction of the antibody response for the whole SOX2 protein sequence using a hidden Markov prediction model. For each region probability scores are calculated. The grey area represents the main 20 mers of target by the SOX2 antibody response in MM patients.

and in none of the MM patients screened, which is in opposition to our results. Here, we have shown that 7.7% of the tested MM patients experience SOX2-specific humoralimmunity. This discrepancy may have at least two different reasons. First, the comparably low number of MM patients included (49 versus 196) may have limited the power of the previous study to detect antibody-positive subjects. Second, we describe herein an association between the application of alloSCT and the development of anti-SOX humoral responses. However, none of the patients described in the previous study had received alloSCT, and, therefore, patients with established MM developing anti-SOX2 antibody responses might have simply been missed.

To the best of our knowledge this is the first study characterizing target epitopes of anti-SOX2 antibody responses. We identified a region of the SOX2 protein which was targeted by the IgG antibodies of the vast majority of seropositive patients. Interestingly, our experimental results were in line with the predictions of an online algorithm [13] naming regions potentially recognized by anti-SOX2 antibody responses. Both approaches described $SOX2_{211-230}$ as an immunodominant epitope. This finding might help to improve SOX2-related immunomonitoring techniques in MM or other diseases such as lung cancer but may also be of use for the design of future immunotherapies targeting SOX2.

AlloSCT induces complex processes in the recipient during which autoantigens and/or tumor-associated antigens may become immunogenic [21, 22]. It is well known that immune responses, in particular T-cell responses, appearing after alloSCT mediate the fatal graft-versus-host disease (GvHD) [23]. On the other hand, donor-derived tumor-specific immune reactions induced by transplantation are central for

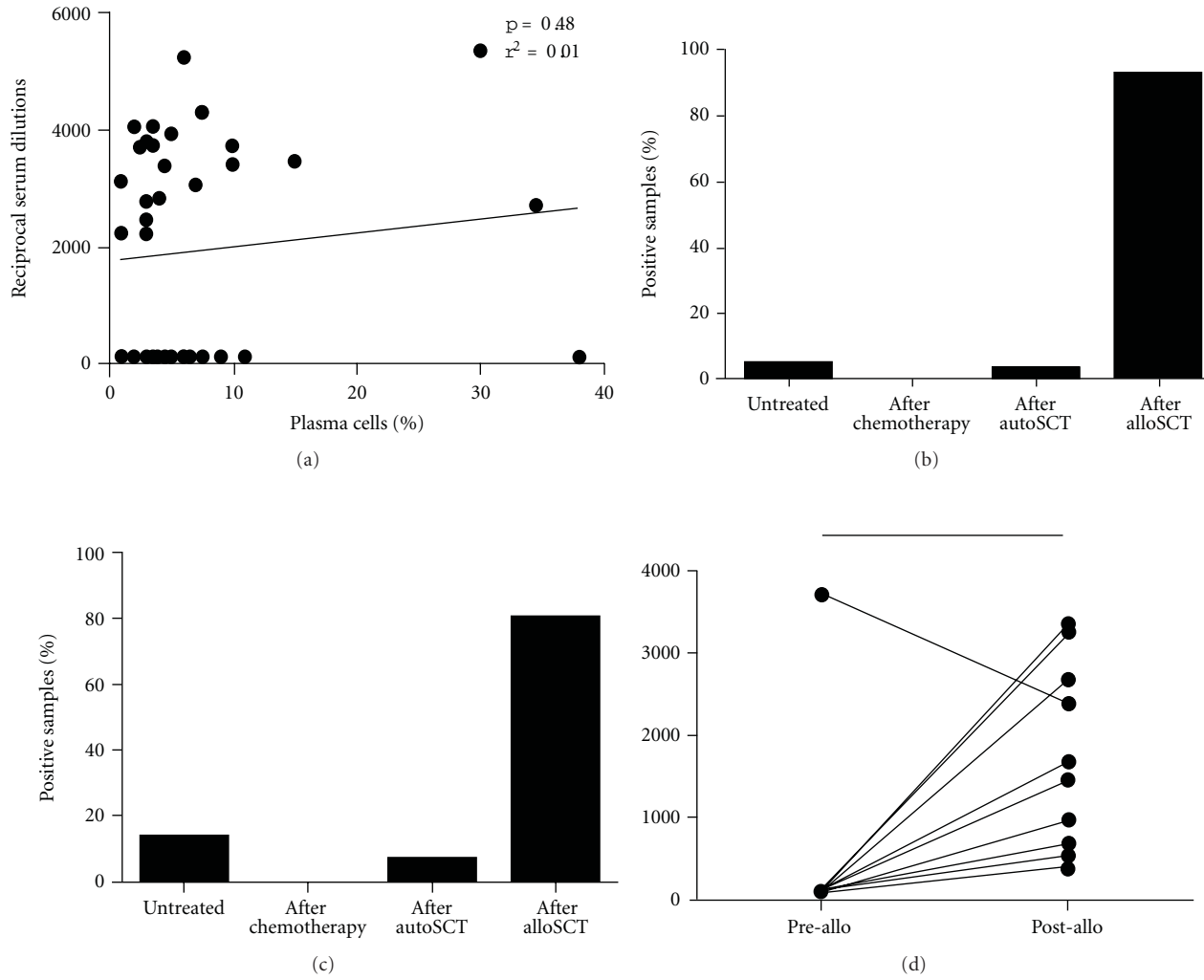

Figure 4: (a) Correlation analysis between the percentage of plasma cells found in the BM of MM patients with the corresponding SOX2-specific antibody titers. For some patients several samples from different time points were analyzed. No significant correlation was found between these two parameters ($P = 0.4826$, $r = 0.01153$). (b) Treatment-dependent distribution of SOX2-positive samples and SOX2-positive patients. 92% (63 of 68) of all SOX2-antibody-positive samples were collected after alloSCT, while 4% (3 of 68) and 2.9% (2 of 68) were collected at time of diagnosis or after chemotherapy, respectively. 80% of SOX2-antibody-positive patients had received alloSCT as maximum treatment, while 13% (2 of 15) and 7% (1 of 15) were untreated or had received autoSCT, respectively. (c) Comparison between SOX2-specific antibody titers before and after alloSCT. Mean values of titers for the respective patient prior and after alloSCT are shown. From 12 SOX2-antibody-positive patients, pre-alloSCT samples were available for 10 patients. 9 of those patients were antibody negative prior to alloSCT and subsequently seroconverted. SOX2-specific antibody titers were significantly higher after alloSCT when compared to pre-alloSCT titers ($P < 0.05$).

the therapeutic potential of alloSCT [24]. Transplantation-induced immunity, in the framework of a graft-versus-tumor effect, is capable of attacking malignant cells. Accordingly, we and others have shown that alloSCT induces immune responses against tumor-specific or overexpressed antigens [12]. In the case of the SOX2 antigen 80% of the seropositive patients had been treated with alloSCT, and most of these patients had been antibody negative prior to transplantation. Thus, we consider it likely that alloSCT might indeed induce SOX2-specific immunity.

What is the biological meaning of the alloSCT-induced SOX-specific immunity? First, anti-SOX2 immune responses might indeed have an (positive or negative) effect on tumor progression. At this time we have no evidence supporting this hypothesis since the occurrence of anti-SOX2 antibodies in our myeloma patients was not related to the disease burden or the remission status. Such an observation would be in line with studies on patients with lung cancer which also failed to detect any association of SOX-specific antibodies with the prognosis of the patients [5, 25, 26].

On the other hand, anti-SOX2 immunity (maybe in concert with immune responses against a multitude of other autoantigens) might simply be a sign of autoimmune or even alloimmune disease, that is, occurring in the framework of

Table 1: Patient characteristics. Data are shown for all patients and for the subgroup of SOX2-seropositive patients. LC: light chain; HC: heavy chain.

Parameter	Total	SOX2 seropositive	Significance
Sex			n.s.
Male	115	9	
Female	80	6	
Age			n.s.
>60	69	6	
≤60	126	9	
Karyotype*			n.s.
Normal	83	7	
Complex	15	0	
del13q14	46	6*	
del17p13	12	3	
$t(4;14)$	9	0	
Not tested	30	0	
LC isotype			n.s.
Light lambda	62	6	
Light kappa	100	7	
HC isotype			n.s.
IgG	167	13	
IgA	18	0	
Stage			n.s.
I	32	2	
II	52	2	
III	95	9	

*One patient was found to bear a 13q14 and a 17p13 deletion.

a GvHD reaction. In our current study, we did not detect an association between the occurrence of GvHD and the presence of anti-SOX2 antibodies. In fact none of the SOX2-antibody-positive patients who had been treated with allo-SCT experienced acute or chronic GVHD.

Since on the one hand SOX2-specific immunity does not seem to be associated with a more favorable course of the disease and on the other hand it does not correlate with a graft-versus-host reaction, the biological meaning of such an immune response remains unclear. We will need to perform prospective studies in MM in cohorts well balanced for treatment and for stage of the disease to understand this immune reaction. Eventually, knowledge gained through such studies will help us to decide whether SOX2 represents a promising prognostic or therapeutic target for patients with multiple myeloma.

Acknowledgments

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Review Article

The Dendritic Cell-Regulatory T Lymphocyte Crosstalk Contributes to Tumor-Induced Tolerance

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Tumor cells commonly escape from elimination by innate and adaptive immune responses using multiple strategies among which is the active suppression of effector immune cells. Regulatory T lymphocytes (Treg) and tolerogenic dendritic cells play essential roles in the establishment and persistence of cancer-induced immunosuppression. Differentiating dendritic cells (DCs) exposed to tumor-derived factors may be arrested at an immature stage becoming inept at initiating immune responses and may induce effector T-cell anergy or deletion. These tolerogenic DCs, which accumulate in patients with different types of cancers, are also involved in the generation of Treg. In turn, Treg that expand during tumor progression contribute to the immune tolerance of cancer by impeding DCs' ability to orchestrate immune responses and by directly inhibiting antitumoral T lymphocytes. Herein we review these bidirectional communications between DCs and Treg as they relate to the promotion of cancer-induced tolerance.

1. Introduction

Despite the arsenal harbored by the immune system to avert tumor development, cancers commonly elude immune detection and elimination by employing multiple strategies [1–5]. The past decade has witnessed considerable advances in our understanding of the mechanisms responsible for the resistance of tumor cells to immune control [6]. These include the downregulation or loss of expression by cancer cells of major histocompatibility complex (MHC) Class I molecules, resulting in the lack of recognition by cytotoxic T lymphocytes (CTL) [6–10]. Resistance to cell death (e.g., expression of antiapoptotic factors, deficiencies in the apoptosis cascade, deficiency in death receptor expression or function, blockade of perforin/granzyme) also contributes to avoidance of tumor cell killing by CTL [5, 11–15]. Additionally, cancer cells may produce immunosuppressive factors that negatively affect the function of DCs, T, and natural killer (NK) cells [11]. Nitric oxide (NO), IL-6, IL-10, tumor growth factor beta (TGF-β), indoleamine 2,3-dioxygensase (IDO), arginase-1, prostaglandin E2 (PGE_2), vascular endothelial growth factor (VEGF), and cyclooxygenase-2 (COX-2) are examples of such molecules that can impede the proliferation and function of $CD4^+$ and $CD8^+$ T cells [5, 12, 16]. This immunosuppressive tumor environment may also foster the generation and/or promotion of immunosuppressive cells such as type 2 macrophages (M2), myeloid-derived suppressor cells (MDSCs), immature/tolerogenic DCs, and Treg [17–20].

By virtue of the immunosuppressive cytokines they secrete or through direct cell-cell contact interactions, both tolerogenic DCs and Treg can block antitumoral T- or NK cell activation and/or induce lymphocyte anergy or apoptosis [20–26]. Such properties place these cells at the center of tumor-induced immunosuppressive networks. Different mechanisms responsible for the accumulation of tolerogenic dendritic cells and Treg in cancer have been described but are still subjected to intensive investigation. One of them may involve a positive feedback loop by which tolerogenic DCs induce Treg that in turn contribute to the induction of immunocompromised DCs. We here review the bidirectional communications between tolerogenic DCs and Treg and

their roles in the context of tumor-induced immunosuppression.

2. The Central Role of Regulatory T Cells and Dendritic Cells in the Induction and Maintenance of an Immunosuppressive Tumor Microenvironment

2.1. Tolerogenic DCs and Their Contribution to Cancer-Induced Immunosuppression

2.1.1. DC Function Depends on Their Maturation and Activation Status. Known for years for their unique capability to function as professional antigen-presenting cells (APCs), DCs play a central role in the initiation and regulation of immune responses and are thereby essential for the protection against infectious pathogens and neoplastic cells [27–30]. DCs are endowed with the potential to activate antigen-specific effector T lymphocytes and are capable of promoting NKT and NK cell function [27, 31, 32]. The efficient stimulation of tumor-specific T lymphocytes by DCs requires the presentation of tumor-derived epitopes on MHC class I and II molecules together with second signals (costimulatory molecules CD80, CD86, CD40) and proinflammatory cytokines such as IL-12 or TNF-α [27, 31–33]. Immature DCs are characterized by high antigen uptake and processing capabilities, but by low expression of costimulatory molecules and thus are not capable of efficiently activating T cells. Multiple DC activation molecules including cytokines (such as interferons, TNF-α, GM-CSF, PGE_2, or IL-1β), ligands of the TNF receptor family, or TLR ligands can act as "danger" signals when tissue damage occurs or pathogens are present [33–35]. These signals promote the differentiation of resident immature DCs into mature DCs characterized by the upregulation of MHC (class I and II) and costimulatory molecules (such as CD80/CD86, OX40L, ICOSL), the production of proinflammatory cytokines including IL-12, TNF-α, IL-1β, or IL-6, and the ability to migrate, in response to specific chemokines, to the secondary lymphoid organs where they encounter naïve T cells [31, 36]. Only fully matured DCs are capable of priming and activating $CD4^+$ and $CD8^+$ T lymphocytes [34, 37, 38]. The ability of DCs to function as inducers of immunity thus depends on their activation/maturation stage.

Although traditionally viewed as the main inducers of immunity, DCs can also participate in the maintenance of peripheral self-tolerance [39, 40]. Under steady-state conditions, in the absence of inflammatory danger signals, immature DCs constantly engulf, process, and present self-antigens from apoptotic cells to potentially self-reactive T lymphocytes, resulting in T-cell anergy or deletion [40–42]. Migration of these immature DCs to the secondary lymphoid organs is contingent upon expression of CCR7, a chemokine receptor normally expressed by mature DCs. This mechanism is essential for the prevention of autoimmunity. In addition to anergizing antigen-specific T cells, these immature DCs have also been involved in the generation of Treg which further contributes to peripheral tolerance [43–46].

2.1.2. Immature/Tolerogenic DCs in Cancer. A profound deficit in the function of DCs (lack of costimulatory molecule expression, decreased production of proinflammatory cytokines, deficiency in the antigen processing and presenting machineries, inability of activating T lymphocytes) has been described in cancer-bearing hosts [26, 47–50]. In cancer patients, tumor-derived factors have been reported to alter DC differentiation and maturation and thereby promote the accumulation of immature DCs (iDCs) in the tumor (tumor-infiltrating DCs, TiDCs) and the lymph nodes. These immunocompromised DCs are unable to initiate antitumor immune responses but can tolerize T lymphocytes [20, 26, 39, 40, 51–54] and, as discussed in Section 3, contribute to the recruitment, expansion, and function of Treg [43, 46, 55–58]. For instance, TiDCs isolated from patients with breast cancer, ovarian cancer, head and neck or lung cancer express inhibitory molecules and fail to induce autologous T-cell proliferation [51, 59, 60]. In murine tumor models a subset of immature myeloid DCs is expanded in the tumor-draining lymph nodes. These immature DCs have decreased production of IL-12, TNF-α, and IL-6 and increased production of IL-10 and TGF-β and of IDO and are responsible for the establishment of an immunosuppressive environment [61]. Upregulation of immunosuppressive molecules such as B7-H4 also contributed to the tolerogenic characteristics of these DCs [62]. Immunocompromised DCs have also been found in rat cancer models. TiDCs expressing MHC class II and ICAM-1 but lacking costimulatory molecules are not capable of inducing allogeneic T-cell proliferation [63–65]. In addition to myeloid iDCs, accumulation of plasmacytoid DCs (pDCs) has also been found in the tumor-draining lymph nodes in B16 tumor-bearing mice [66] and in head and neck human tumors [67]. These pDCs are recruited to the tumor microenvironment in response to several chemokines, including CCL20, stromal cell-derived factor-1/CXCL12, and Ag-5/vascular cell adhesion molecule-1 interactions [68, 69]. The majority of these pDCs exhibit poor immunostimulatory capacity, express IDO, and may promote $FoxP3^+$ Treg rather than activating effector T lymphocytes [70, 71]. In humans, the accumulation of IDO-expressing cells in melanoma [72–74], pancreatic ductal adenocarcinoma [75], ovarian cancer [76], colon cancer [77, 78], and non-small-cell lung cancer [79] has been associated with a worsened clinical outcome. However, in contrast to these observations, IDO expression in tumor endothelial cells of patients with renal cell carcinoma seems to restrict tumor growth and to contribute to long-term survival, possibly by limiting the influx of tryptophan from the blood to the tumor or by generating metabolites toxic to tumor cells [80]. These opposite results may be explained by the type of cells expressing IDO. In fact, unlike other malignancies where the main source of IDO is either the cancer cells themselves or tumor infiltrating leukocytes (DCs, eosinophils), in renal cell carcinoma IDO is almost exclusively expressed by endothelial cells of newly formed blood vessels. IDO expression by cells involved in the microvasculature has been associated with a Th-1-related cytokine milieu (mainly IFN-γ) [80] which may impair tumor growth. Consistently, high microvessel density correlates with lower tumor grade and prolonged

survival of patients with renal cell carcinoma [81]. Immature/tolerogenic DCs may also contribute to tumor development by fostering tumor angiogenesis. They are indeed capable of producing different cytokines and growth factors such as VEGF, promoting neoangiogenesis [82, 83].

Different approaches have been evaluated to correct the phenotypical and functional deficiencies of DCs in cancer, which include attempts to promote their maturation using different techniques. For example, the combination of CpG and anti-IL-10R antagonist has been reported to enhance IL-12 production and therefore the capacity of DCs to activate specific T cell *in vitro* and *in vivo* [84]. Interestingly, short-term ablation of DCs *in vivo* using a diphtheria toxin-based system has been reported to impair tumor growth in animal models [85].

Tumors have developed a series of strategies to suppress DC function. Some of the defined mechanisms underlying the blockade of DC maturation and the accumulation of tolerogenic DCs include the production of immunosuppressive factors such as TGF-β, IL-10, IL-6, VEGF, IDO, and PGE_2 [11, 18, 70, 86]. This results in the induction of inhibitory signaling pathways in DCs. One of them involves the transcription factor STAT-3, which plays a key role in the regulation of inflammatory processes [87]. Constitutive STAT-3 activation in tumors (both of hematopoietic and of epithelial origin) inhibits the production of proinflammatory cytokines by infiltrating immune cells while promoting the release of soluble factors that suppress DC function [87–92]. Furthermore, some tumor-derived molecules (VEGF, IL-6) enhance the expression of STAT-3 in DCs [20, 91, 92]. STAT-3 activation, although an important event in early differentiation of DCs, is decreased in fully differentiated mature DCs [91]. Tumor-induced maintenance of constitutive STAT-3 activation in DCs eventually results in the acquisition of the tolerogenic potential of these cells [91, 93–98]. Expectedly, the disruption of STAT-3 signaling, for example, using dominant negative STAT-3 variants in the mouse, leads to tumor regression or growth control *in vivo* [90, 98, 99]. Similarly, the cytokine signaling inhibitor SOCS-1 has been highlighted as an important regulator of DC APC function [100]. The inhibition of this molecule using specific siRNA has been reported to break tolerance to the self-antigen Trp2 in an established B16 tumor model [100].

In addition to the mechanisms described above, tumor-induced Treg may also participate in the inhibition of DC maturation and thus in the generation of tolerogenic DCs.

2.2. Regulatory T Cells Critically Contribute to Tumor-Induced Tolerance

2.2.1. Regulatory T Lymphocytes. Initially described in the field of autoimmunity, regulatory T cells (Treg) are comprised of a heterogeneous population of T lymphocytes defined by their capacity to suppress immune responses to self- and foreign antigens [23, 101–105]. Treg can act as critical checkpoints in the control of autoimmunity, infections, or cancer [19, 23, 101, 106–110]. A wide diversity of immunosuppressive T cells have been identified [101]. As a member of the growing family of immunosuppressive/regulatory T lymphocytes [23, 101, 107], the $CD4^+CD25^+$ Treg subset has been extensively studied over the last two decades. These cells constitute about 10% of the circulating T-lymphocyte population in mice and 5% in healthy humans [111]. In addition to CD25, the α-chain of the IL-2 receptor, this lymphocyte subpopulation also expresses multiple markers including cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR), CD62L, lymphocyte activation gene 3 (LAG 3), Toll-like receptors (TLR-4, -5, -7, -8) [112]. In human, the IL-7 receptor (CD127) has been used to distinguish Treg from activated T cells. CD127 expression has indeed been reported to inversely correlate with FoxP3 expression and the suppressive function of Treg [113, 114]. However, increased CD127 expression has also been detected on activated (ICOS- and CD103-expressing) Treg subsets [115]. Expression of the ectonucleotidase CD39 by $FoxP3^+$ Treg has been reported in mouse and human [116]. However, in contrast to mice, in human this enzyme seems to be restricted to a subset of $FoxP3^+$ regulatory effector/memory-like T (Trem) cells [116]. CD39 together with another ectoenzyme (CD73) is involved in the generation of pericellular adenosine from extracellular nucleotides, resulting in the suppression of adenosine A2A receptor-expressing activated T-effector cells [117]. The forkhead/winged helix transcription factor FoxP3 appears fundamental for the development and function of $CD4^+CD25^+$ Treg and remains the most specific molecular marker for these cells [112, 118–121]. Treg contribute to the prevention of autoimmune diseases by controlling the activity of autoreactive T lymphocytes that have escaped negative selection in the thymus [103, 105, 122]. Elimination of Treg or genetic alteration of the FoxP3 gene results in the development of lethal autoimmune conditions, evidencing the essential role of these cells in the maintenance of active dominant peripheral tolerance [111, 123–125]. Depending on their origin, two types of $CD4^+CD25^+FoxP3^+$ Treg can be identified. Naturally occurring Treg (natural or nTreg) that develop in the thymus and adaptive (inducible or iTreg) are generated by the conversion of $CD4^+CD25^-$ naïve T cells in the periphery [126–128]. It has been documented that Treg survival and immunosuppressive function and Treg production from naïve T cells depend on external signals, some of which are relayed by the TCR, CD28, TGF-β, and IL-2 receptors and other yet to be identified molecules [101–103, 129–132], converging towards the regulation of specific gene expression such as FoxP3. Although most iTreg are characterized by a $CD25^{high}$ phenotype, the generation of $CD25^-$ Treg by coimmunization with highly antigenic epitopes has also been reported [133]. In addition, the significance of CD25 expression by Treg is subjected to discussion, and T cells with regulatory properties have also been detected in the $CD4^+CD25^-$ subset [134–136]. The cellular and molecular bases for the suppressive activity of $CD4^+CD25^+$ Treg cells remain contentious [101, 119, 137–140]. Some proposed mechanisms include the production of inhibitory cytokines such as IL-10, TGF-β, and IL-35, a direct cell contact involving CTLA-4 and CD80/CD86, expression of granzymes, the depletion of IL-2 from the environment,

the transfer of cAMP to the target cells, the release of nucleosides, and other yet unidentified mechanisms [23, 138, 141–148].

2.2.2. Role of Treg in Cancer. Multiple studies have demonstrated that, besides their role in autoimmunity, Treg critically contribute to the immune tolerance of cancer. An increase in the number of these cells has been detected in the blood, lymph nodes, and spleen of tumor-bearing hosts and correlates with poor prognosis [24, 48, 127, 149–153]. Treg expansion observed during tumor progression may result from the proliferation of nTreg or from the conversion of $CD4^+CD25^-FoxP3^-$ T cells into $CD4^+CD25^+FoxP3^+$ iTreg [19, 126]. These two mechanisms may be complementary and may act in concert to achieve an optimal Treg expansion as reviewed in [102, 103, 111, 154]. In addition, it has been documented that a variety of tumors including breast cancer, melanoma, and lymphoma may recruit Treg to the tumor site. This Treg recruitment may involve a CCR4-dependent trafficking induced by CCL22 released by tumor cells and immune cells infiltrating the tumors such as macrophages and DCs [155]. This attraction of Treg by cancer cells and the modulation of Treg trafficking by tumor may be an essential element for the accumulation of Treg in the tumor microenvironment and for the mode of action of these cells in cancer [19, 106, 120, 127, 151, 156–159]. Treg impede antitumoral immune responses by suppressing the function of effectors $CD4^+$, $CD8^+$, and NK cells [24, 160–164] and also by inhibiting DC activation [48, 144, 165–168] as discussed in Section 4.

Since Treg represent a major obstacle for the elimination of tumors by immune cells, their therapeutic depletion or their functional inactivation using drugs or antibodies has been shown to improve responses to cancer immunotherapy including DC-based vaccines [150, 163, 169–171]. Different strategies have thus been explored to deplete/inactivate Treg *in vivo* [150, 163, 169–186]. However, the selective elimination or inactivation of Treg still constitutes a major challenge in immunotherapy since these cells share the same surface markers as activated conventional nonsuppressive T cells. Antibody-based approaches indistinctly target both Treg and activated effector T lymphocytes, and in most cases chemotherapeutic agents used to eliminate Treg do not exert specific effects on these cells. We have shown in the rat that cyclophosphamide administration results in elimination of both regulatory and effector T cells but that effector cell reconstitution occurs earlier than that of Treg [150]. Cyclophosphamide therapy enhanced tumor-specific vaccination [150]. At a low dose cyclophosphamide has been shown to trigger apoptosis of mouse Tregs *in vitro* and *in vivo* without significant changes in $CD4^+CD25^-$ cell viability [183, 187, 188]. However, clinical studies have also indicated that cyclophosphamide may not significantly affect Treg number and function [189]. Elimination of Treg based on CD25 expression results in the concurrent depletion of activated effector lymphocytes [154]. In addition, this strategy may foster tumor-driven conversion of Treg from $CD4^+CD25^-FoxP3^-$ T cells [154, 185].

3. Promotion of Treg Expansion and Function by DCs

The mechanisms controlling the induction and maintenance of Treg during tumor development are still being elucidated. As outlined above, although critical for the development of adaptive immune responses, DCs may also contribute to the mechanisms of immune tolerance. These "tolerogenic" DCs of both plasmacytoid (pDCs) or myeloid (mDCs) origin are not only capable of anergizing effector T lymphocytes but may also be endowed with the capacity to drive the differentiation and/or proliferation of $FoxP3^+$ Treg [39, 43, 46, 53, 58, 67, 190–199]. The ability of DCs to induce immune tolerance is believed to depend on their origin, activation state, the nature of the maturation signals and the cytokine context at the time they encounter T lymphocytes. Different subsets of tolerogenic DCs capable of promoting Treg expansion and/or function have been described [53, 57, 192, 195, 199, 200]. In physiological conditions, steady-state immature myeloid DCs constantly engulf and process self-antigens and upon migration to the draining lymph nodes can block self-reactive effector T cells and promote Treg expansion [39, 40, 58], thus contributing to the prevention of autoimmunity. In addition, semimature myeloid DCs, which exhibit some of the characteristics of mature DCs (including costimulatory molecule expression) but that produce significantly lower level of proinflammatory cytokines, have also been described for their ability to drive the differentiation of adaptive Treg [20, 39, 55, 196, 201, 202]. Importantly, phenotypically mature DCs not only induce immunity but may also exhibit a tolerogenic function. For instance, DCs isolated from Peyer's patches, lungs, or the anterior chamber of the eye display a mature phenotype, secrete IL-10, and are capable of inducing Treg [200]. CD40L-activated pDCs may also be tolerogenic and support Treg expansion [43, 203]. In addition, following extensive stimulation *in vitro* with maturation signals (e.g., LPS), DCs become "exhausted" and produce IL-10 but not IL-12 and elicit nonpolarized memory cells and/or Th2 responses [204]. Whether these "exhausted" DCs may also induce Treg *in vivo* remains however to be determined. In addition, variable results have been reported as to whether mature or immature DCs may preferentially lead to Treg induction [55, 200].

The mechanisms underlying DC-mediated induction of Treg are still not entirely clear. Evidence has been provided that IDO, a key-enzyme that catalyses the degradation of the essential amino acid tryptophan into kynurenine, may play an important role in this process [70, 205]. IDO-mediated tryptophan deprivation from the T-lymphocyte environment results in the downregulation of TCR-ζ-chain and leads to the activation of the GCN2 (general control nonrepressed 2) kinase pathway that prevents T-cell cycling and activation [206, 207]. In addition the byproducts of the tryptophan catabolism such as L-kynurenine, 3-hydroxykynurenie, or 3-hydroxyanthranilic acid may be endowed with inherent suppressive activity [206, 207]. IDO can be expressed by different DC subsets in mouse and human [208]. Although $CD8^+$ DCs and plasmacytoid DCs were originally identified as the main source of IDO, it has recently been shown that $CD8a^-$ IDO^-

DCs can be converted into IDO$^+$ tolerogenic DCs [209]. IDO expression has been identified as a possible factor involved in DC-mediated induction of Treg [66]. In mice and human it has been reported that IDO$^+$ DCs are able to promote the differentiation of iTreg from a pool of naïve T cells [206–208, 210]. Treg induction and activation by IDO$^+$ DCs require the GCN2 pathway and may be prevented by CTLA-4 blockade [66]. It has also been shown that the production of TGF-β by DCs conditioned by the tumor microenvironment also promotes iTreg generation [126]. TGF-β, together with TCR and CD28 ligation, induces an intracellular signaling that involves the cytosolic Smad proteins (Smad 2 and 3) and STAT-3 and -5 activation, resulting in FoxP3 expression [112, 118, 126, 211]. Engagement of T-cell CTLA-4 and GITR by their ligands on DCs induces the activation of preexisting Treg as well as their *de novo* generation [66, 156, 208, 210]. The engagement of programmed death receptor-1 (PD-1) expressed by T cells with B7-H1 expressed by DCs and macrophages results in the negative regulation of target T lymphocytes [212]. B7-H1-expressing DCs generated in the tumor environment exhibit reduced T-cell stimulatory capacity and have been reported to foster Treg expansion by conversion of naïve T cells into iTreg and/or by promoting the proliferation of nTreg [212–215].

The homing of Treg to the tumor site or to the tumor-draining lymph nodes where they interact with their targets is essential for their role in cancer-induced tolerance. DCs are capable of modulating the trafficking and therefore the recruitment of Treg to the tumor site or to the secondary lymphoid organs [44, 155, 216]. Blood Treg have been shown to express high CCR4 and to selectively migrate in response to the CCR4 ligand CCL22 produced by tumor cells but also by tumor infiltrating DCs [127, 217–221].

In summary, DCs subverted by the tumor microenvironment lack effector T-cell stimulatory capacity but are endowed with the ability to promote suppressive Treg. In addition to tumor-derived factors which can directly induce Treg proliferation and/or generation from naïve T cells, DCs that differentiate in the tumor microenvironment provide essential signals that contribute to Treg expansion. Induction of Treg by DCs thus appears as one essential mechanism employed by cancers to generate immunosuppressive Treg and thereby to escape from antitumor immune responses (Figure 1).

4. Treg Negatively Modulate DC Maturation and Promote the Generation of Tolerogenic DCs

These interactions between immunosuppressive/tolerogenic DCs and Treg are not unidirectional, and Treg can "talk back" to DCs, influencing their maturation status (Figure 1). In a nontumor setting, the downregulation of DC costimulatory molecule expression [144] and IL-12 secretion [167] by Treg has been documented in the mouse. Human Treg have also been reported to exhibit suppressive effects on monocyte/macrophages [168] and on DCs generated from peripheral blood monocytes [166]. An inhibition by Treg of the maturation induced by a cocktail of TLR ligands of human myeloid but not plasmacytoid DCs has also been reported [222]. Other studies have indicated that Treg may suppress DC costimulatory molecules CD80 and CD86 without affecting CD40 expression and that inhibition of DC maturation occurs in the absence of CD40-CD40L interaction [198]. In tumor immunity, Treg have primarily been described for their ability to impair the function of tumor-specific CD4$^+$ and CD8$^+$ T cells [102, 106, 223]. However, it has been reported that Treg from tumor-bearing mice may impair the expression of DC costimulatory molecules CD80, CD86, and CD40, suppress DC production of proinflammatory cytokines IL-12 and TNF-α, and inhibit their ability to induce T-cell activation *in vitro* [48, 165]. A proposed mechanism underlying tumor-induced Treg-mediated suppression of DCs may involve the suppressive cytokines TGF-β and IL-10 [48].

Treg have also been reported to induce the expression of the immunosuppressive molecules B7-H3 and B7-H4 on DCs [44, 224–226]. B7-H3 and B7-H4 are members of the B7 family, but, in contrast to their activating counterparts, they trigger inhibitory signals in T lymphocytes and thus contribute to the immunosuppressive function of DCs and thereby to cancer-induced tolerance [44, 212, 225]. These modifications in the expression of DC surface markers may depend on diverse mechanisms, and, in addition to CTLA-4, a role for LFA-1 (lymphocyte function-associated antigen 1), LAG-3 [227], and neuropilin-1 has been proposed [227]. The engagement of the B7 molecules on DCs by CTLA-4 on Treg has been shown to upregulate IDO production in human and murine DCs which then promote Treg [206]. In turn, IDO-activated Treg have been shown to induce PD-L1 upregulation on DCs [66, 207] resulting in an efficient feedback amplification loop [66]. An additional mechanism by which Treg may promote tolerogenic DCs involves the induction of IL-10 production by DC [226].

Importantly, mature DCs have been shown to be refractory to Treg-mediated inhibition and seem to display a stable phenotype when exposed to these suppressive cells [144, 222]. Mouse bone-marrow-derived DCs first activated with the TLR4 ligand LPS and exposed to tumor-induced Treg maintain expression of CD80, CD86, and CD40, produce IL-12 or TNF-a, and are not impaired in their allostimulatory activity [48]. This resistance of mature DCs to Treg suppression has therapeutic implications as it underlines the importance of activating *in vitro* DCs used as vaccines prior to their administration.

Thus, Treg contribute to tumor-induced tolerance by restraining DC maturation, proinflammatory cytokine production, and APC function, therefore participating in the induction and accumulation of tolerogenic DCs.

5. Conclusion

There is clear evidence that DCs rendered tolerogenic by the immunosuppressive tumor microenvironment are capable not only of inhibiting effector antitumoral T cells but also of promoting the differentiation of iTreg from naïve T lymphocytes or of fostering the proliferation of nTreg. Reciprocally,

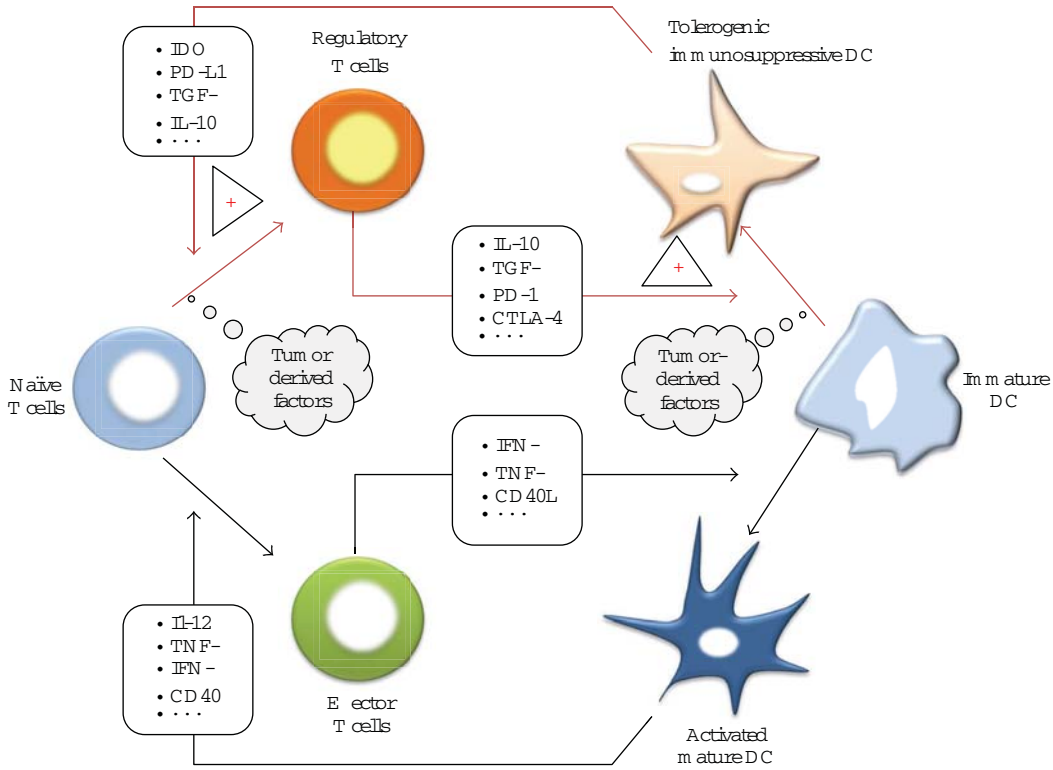

Figure 1: Bidirectional communications between Treg and tolerogenic DCs in cancer. Tumor-derived factors can promote the differentiation of immature DCs and naïve T cells into tolerogenic DCs and Treg. Tolerogenic DCs contribute to the generation of Treg by various mechanisms. In turn, Treg participate in tumor-induced tolerance by restraining DC maturation and fostering the accumulation of tolerogenic DCs.

cancer-induced Treg, by restraining DC maturation and by inducing DC expression and production of immunosuppressive molecules, may skew their differentiation towards a tolerogenic cell population. This positive feedback loop by which suppressed/tolerogenic DCs may induce Treg that in turn enhance DC immune inhibitory function may significantly contribute to the persistence of the immune tolerance to cancer.

These DC-Treg interactions, by enhancing tumor-induced immunosuppression, represent a major barrier to successful immunotherapy. Therefore, targeting the generation of these two suppressive cell populations is a desirable goal in chemo- and immunotherapeutic approaches. To achieve this objective there is a need to further improve strategies to simultaneously promote the full activation of DC using selective adjuvants such as TLR ligands or cytokines and impair Treg expansion, function, and recruitment.

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Review Article

Toll-Like Receptor 4 Activation in Cancer Progression and Therapy

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Cancer immunotherapy has been the focus of intense research since the late 19th century when Coley observed that bacterial components can contribute to cancer regression by eliciting an antitumor immune response. Successful activation and maturation of tumor-specific immune cells is now known to be mediated by bacterial endotoxin, which activates Toll-like receptor 4 (TLR4). TLR4 is expressed on a variety of immune as well as tumor cells, but its activation can have opposing effects. While TLR4 activation can promote antitumor immunity, it can also result in increased tumor growth and immunosuppression. Nevertheless, TLR4 engagement by endotoxin as well as by endogenous ligands represents notable contribution to the outcome of different cancer treatments, such as radiation or chemotherapy. Further research of the role and mechanisms of TLR4 activation in cancer may provide novel antitumor vaccine adjuvants as well as TLR4 inhibitors that could prevent inflammation-induced carcinogenesis.

1. Introduction

Immune system plays a crucial role not only in defense against microbial infection but also in control and surveillance of malignant neoplasms. Immune cells scan tissues with the objective to remove newlyformed malignant cells before they turn into fully formed tumors. Malignant cells developed intricate mechanisms that enable them to inhibit immune cells through secretion of specific cytokines that create an immunosuppressive environment [1]. Tumors can even directly kill tumor-infiltrating lymphocytes, which are CD95 sensitive, by expressing the CD95L (Fas ligand) [2].

Innate immunity is the first line of defense against microbial infection. Innate immune cells recognize the intruding pathogen and trigger appropriate immune response with the help of Toll-like receptors (TLRs), arguably the most important vertebrate innate immune receptors. TLRs recognize different molecules of microbial origin, called pathogen-associated molecular patterns. TLRs are located on the cell surface (TLR1, 2, 4, 5, 6) or in the endosomal compartments (TLR3, 7, 8, 9), where they safeguard the organism against infection. After recognition of their respective ligands, TLRs dimerize and trigger a cytoplasmic signaling pathway that leads to activation of several nuclear factors (e.g., NFκB, IRF) responsible for transcription of immune genes [3].

Toll-like receptor signaling in immune cells is critical for regulation of innate and adaptive immune responses, such as DC maturation and antigen presentation as well as CD8+ T-cell cytotoxicity, all of which are important factors in antitumor immunity [4]. On the other hand, TLR stimulation can also result in enhanced regulatory T-cell proliferation and suppressor function favoring tumor development [5–7]. TLR expression is not limited to immune cells, and indeed many tumor cells have been found to express TLRs, signaling through which can promote tumor growth and immune evasion [8, 9]. On the other hand, TLR signaling in tumor cells was also shown to reduce the proliferative capacity of tumor cells [10]. We will focus

on reports concerning TLR4 signaling and its involvement in cancer development and progression as well as the therapeutic benefit that could come from TLR4 stimulation.

2. Toll-Like Receptor 4 in Health and Disease

TLRs are homologues of Toll, a receptor found in insects, that is involved in establishing dorsoventral polarity during embryogenesis as well as in immune response against fungal infections [11, 12]. The first discovered human Toll homologue was TLR4. It recognizes endotoxin (i.e., lipopolysaccharide), an outer membrane component of Gram-negative bacteria, that is composed of a conserved amphipathic lipid A component and of variable polysaccharides. The mechanism of TLR4 activation is quite complex and (unlike other TLRs) involves several auxiliary proteins (LBP, CD14) as well as a coreceptor (MD-2) [3] (Figure 1). It is in fact MD-2 and not TLR4 that directly recognizes and binds endotoxin [13, 14]. MD-2 is a soluble protein with a large hydrophobic pocket that represents the binding site for the acyl chains of lipid A. Lipid A is usually composed of 6 acyl chains, but only 5 of them bind into the hydrophobic pocket of MD-2. The 6th acyl chain protrudes out of the pocket and interacts with hydrophobic residues on TLR4. These interactions are crucial for MD-2/TLR4 heterodimerization and therefore prerequisite for the activation of the TLR4 signaling cascade [15, 16]. The endotoxin/MD-2/TLR4 heterodimer can, unlike other TLR signaling complexes, recruit two distinct intracellular adaptor proteins (i.e., MyD88/TIRAP and TRIF/TRAM) and can therefore activate two parallel signaling pathways and trigger the transcription of both proinflammatory cytokines as well as type I interferons [3]. Immune effects of TLR4 activation are indeed extensive; LPS alone can activate over 1000 genes [17]. It is therefore not too surprising that TLR4 activation affects not only the immune response against invading Gram-negative bacteria but is also involved in chronic inflammation, autoimmune diseases, and malignancies. TLR4 signaling in cancer is considered a double-edged sword. If TLR4 is activated on immune cells, it can enhance anti-tumor immunity. On the other hand, chronic inflammation is a major risk factor in cancer development [18].

3. TLR4 Expression in Cancer Cells

Progress in cancer research over the past decade has been immense, and the original fundamental characteristics of cancer (sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, invasion, and metastasis) [19] have recently been revisited and updated. Evasion of immune destruction rises as a new emerging hallmark of cancer [20]. Tumors utilize multiple mechanisms that help them turn the immune balance in their favor. They can secrete immunosuppressive cytokines (TGFβ, IL-10, etc.), express antiapoptotic molecules, or downregulate tumor antigens and MHC1 expression [1]. TLRs are expressed by a variety of tumor cell lines, both in mouse and in human

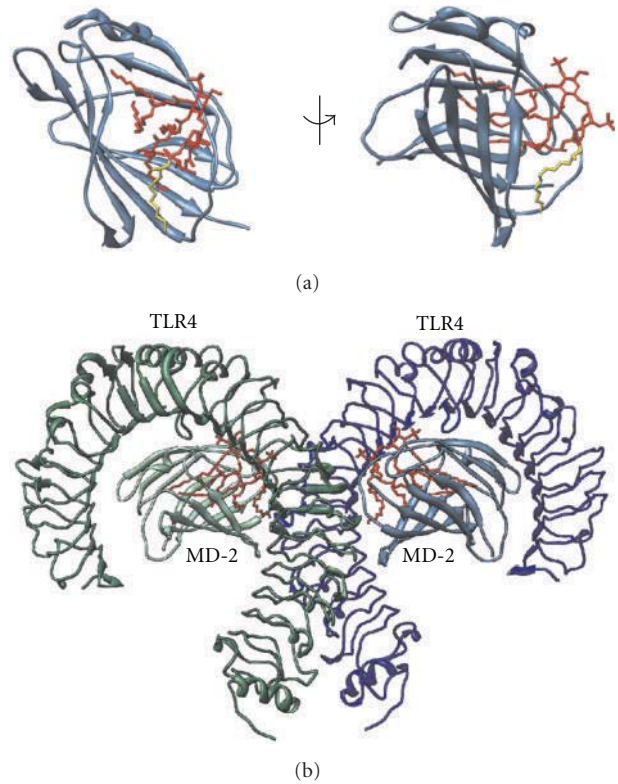

Figure 1: TLR4/MD-2 receptor complex recognizes and binds endotoxin. (a) MD-2 (shown in blue ribbons) is a soluble protein with a large hydrophobic pocket that directly binds bacterial endotoxin (red). One of the acyl chains of endotoxin (yellow) remains outside the hydrophobic pocket and mediates crucial interactions with TLR4 that bind the TLR4/MD-2 heterodimer together. Left: direct view of the MD-2 hydrophobic pocket. Right: side view showing the protruding endotoxin acyl chain. (b) The TLR4/MD-2/endotoxin heterodimer. Only the extracellular domains of TLR4 whose crystal structures were determined are shown [16].

Table 1: Murine tumor cell lines that express TLR4.

Tumor type	Murine tumor cell line	References
Breast cancer	4T1	[8]
Colon cancer	MC26	[8]
Glioma	GL261	[21]
Lung cancer	LLC1	[8]
Melanoma	B16	[8]
Prostate cancer	RM1	[8]

(Tables 1 and 2). Many of them are not limited to a single TLR but rather utilize an assortment of different TLRs (similarly to immune cells).

Expression of TLR4 was confirmed by RT-PCR and FACS analysis on a large number of murine tumor cells, such as colon, breast, prostate, lung, and melanoma cancer cells. TLR4 signaling was shown to be unimpaired and could induce the synthesis of soluble immune mediators that could help the tumor to withstand the immune attack [8]. MC26 cells, for example, were shown to express functional TLR4 that (when activated by endotoxin) triggered

Table 2: Human tumor cell lines that express TLR4.

Tumor type	Human tumor cell line	References
Bladder cancer	T24	[22]
Breast cancer	MDA-MB-231	[23]
Colon cancer	SW480, HT29, KM20	[24, 25]
Laryngeal and oral cancer	PCI-1, PCI-30	[26]
Melanoma	SkMEL-28, BN1, 9923M, ME5, ME16, ME17	[27, 28]
Neuroblastoma	NB-1	[29]
Ovarian cancer	SKOV3, AD10, A2780, CP70	[9, 30, 31]

activation of NF-κB, ERK, and JNK kinases as well as the synthesis of iNOS, IL-6, and IL-12p70 [8]. iNOS and IL-6 have immunosuppressive effects [32–34], but IL-12p70 is generally not considered favorable for tumor development since it activates NK cells, induces T-cell proliferation, and promotes specific allogenic CTL reactions [35]. Some papers indicate that IL-12p70 can also have suppressive effects on allogenic or tumor-specific CTL generation [36, 37], but since evidence undisputedly demonstrates anti-tumor effects for IL-12 its production by tumor cells is possibly just a side product of TLR4 activation and subsequent NF-κB activation.

Supernatants from endotoxin-stimulated tumor cells were shown to inhibit T-cell proliferation and NK-cell cytotoxicity. Furthermore, blockade of tumor TLR4 signaling with anti-TLR4 siRNA or with inhibitory TLR4 peptide treatment prolongs the survival of MC26-bearing mice [8].

Functional TLR4 signaling was also demonstrated on human tumor cells. On colon carcinoma cells TLR4 signaling, in addition to production of immunosuppressive factors, also improved tumor cell apoptosis resistance [24]. Moreover, endotoxin stimulation of human prostate epithelial cancer cells elicited production of immunosuppressive and proangiogenic factors (TGF-beta and VEGF, resp.) [38].

TLR4 is expressed not only on malignant cells but also on normal tissues and benign tumors [30, 31]. Much remains to be studied concerning the function of TLR4 on normal nonimmune tissues in correlation with cancer development. But we must not forget to examine the expression of other contributing proteins in the TLR4 signaling cascade, for example, the adapter protein MyD88 (myeloid differentiation 88) that is essential for pro-inflammatory signaling. Although TLR4 expression was shown in normal ovarian epithelium, MyD88 was not expressed, therefore rendering TLR4 signaling via the proinflammatory MyD88-dependent pathway nonfunctional [9, 30]. Similar observation was made in a variety of colorectal carcinoma cell lines where tumor cells expressed TLR4 but failed to coexpress CD14, an important auxiliary protein in the endotoxin receptor complex [39] (Table 3).

Table 3: Human tumors expressing TLR4.

Tumor type	References
Adrenocortical cancer	[40]
Breast cancer	[41]
Bladder cancer	[22]
Colon cancer	[24, 25, 39]
Gastric cancer	[42]
Laryngeal cancer	[26]
Lung cancer	[43]
Melanoma	[27, 28]
Neuroblastoma	[29]
Ovarian cancer	[9, 30, 31]
Prostate cancer	[44]

4. Chronic Inflammation Mediated by TLR4 in Cancer Development and Progression

Numerous links exist between inflammation and tumor development [18]. At the same time inflammatory cytokines are indispensable for immune cell activation and antitumor function. Therefore, there is an apparent contradiction when we consider the role of inflammation in cancer. It is plausible that part of the answer to this puzzle lies not in the inflammatory stimulation *per se* but in its timing, duration, and intensity.

Chronic inflammation is often associated with cancer and can be the result of different causes, such as autoimmune disease or microbial infection.

An example of microbial infection that can predispose an individual to cancer development is *Helicobacter pylori* infection. Infection with *H. pylori* is a known risk factor in gastric cancer and has been classified as a human carcinogen by the International Agency for Research on Cancer [45]. *H. pylori* infection is chronic and persistent, because *H. pylori* has the ability to evade immune system recognition. It has unusual endotoxin that exhibits very low endotoxic activity compared to the more common hexa-acylated form of endotoxin, usually found in enterobacteria (e.g., *Escherichia coli*) [46]. In spite of the inability to stimulate TLR4 on its own, *H. pylori* actively promotes inflammation by upregulating TLR4 expression via TLR2 and MEK1/2-ERK1/2 pathway giving way to TLR4 activation by endotoxin from other bacteria that pass through the gastrointestinal tract [47, 48]. TLR4 expression was indeed observed on gastric carcinoma tumor cells as well as on gastric epithelium with intestinal metaplasia and dysplasia [42].

Persistent inflammation is also a characteristic of colitis-associated neoplasms. Patients with ulcerative colitis have a

five to eight times higher risk of developing colorectal cancer than the rest of the population [49, 50]. TLR4 expression is upregulated in colitis-associated cancer lesions from patients with ulcerative colitis but not in the surrounding tissue [51]. TLR4 seems to promote the development of colitis-associated colorectal tumors, and mice deficient in TLR4 are markedly protected against the development of neoplasia [52]. The reason behind this phenomenon could lie in the TLR4-Cox2-PGE_2 signaling axis. Cyclooxygenase-2 (Cox-2) is aberrantly expressed in the majority of colorectal tumors and is (along with its enzymatic product prostaglandin E_2) involved in the development of colorectal cancer [53]. It was recently shown that oral administration of high dosages of PGE_2 can by-pass the protective effect exhibited by TLR4-deficient mice, which implicates PGE_2 as an important TLR4 downstream molecule in colorectal cancer development as well as a potential target for more effective prevention of colitis-associated colorectal cancer [54].

TLR4 also has the potential to become a disease progression marker in patients with colon cancer or premalignant lesions [55] as well as a biomarker of the aggressive tumor phenotype in laryngeal carcinoma and breast cancer [41, 56]. Its high expression correlates with poor prognosis in colorectal cancer patients [57] and in murine models [58]. Furthermore, TLR4 is associated with liver metastasis; researchers showed an increase in TLR4 expression in steatotic murine livers following diet-induced obesity. In a metastatic model of colorectal cancer animals with steatotic livers had increased metastatic tumor mass within the liver compared to lean controls. Silencing of TLR4 on tumors lowered the tumor burden, indicating that tumor cell TLR4 signaling promotes metastatic growth [58]. On the contrary other studies concerning colorectal carcinoma showed correlation between reduced TLR4 expression and increased metastatic potential of the tumor [39].

TLR4 is associated with metastasis also in other types of cancer, such as melanoma, where TLR4 activation induces cell migration [28], and prostate cancer. It was shown that highly metastatic human prostate cancer cell lines, such as PC3 or DU145, express higher levels of TLR4 compared to poorly metastatic cell lines. Moreover, downregulation of TLR4 expression by siRNA can inhibit prostate cancer cell invasion *in vitro* and can improve survival of tumor-bearing animals [59]. Similar results were shown in human breast cancer cell line, where downregulation of TLR4 significantly reduced tumor cell proliferation [23].

Conversely, another study [60] reports a decrease in TLR4 expression in human prostate tissue samples that correlates with histopathological grade of prostate cancer. TLR4 expressed in normal and low-grade tumors could therefore be a contributing factor in chronic inflammation that promotes carcinogenesis [61], while decreased TLR4 expression in more aggressive high-grade tumors could result from loss of cell differentiation that accompanies cancer progression [60].

A similar phenomenon, though with a different underlying cause, can be seen in the case of cervical cancer, where Yu and coworkers [62] observed downregulation of TLR4 expression during progression of cervical neoplasia. They have attributed this downregulation to the immunosuppressive effect that persistent human papilloma virusinfection has on the host immune response [62]. A degree of prudence is therefore recommended when conclusions are made from the data currently available, because of major discrepancies between studies with respect to different species, cell culture, or cancer type studied.

5. Endogenous TLR4 Ligands Responsible for TLR4 Signalization in Cancer

But what activates TLR4 signaling—is it bacterial endotoxin or perhaps other ligands? Endotoxin is ubiquitously present in air, gut, and epithelial surfaces, and perioperative exposure to it is associated with accelerated metastatic tumor growth [63]. Metastases could be the consequence of activation of the TLR4 signaling pathway that results in reduced apoptosis and increased proliferation of metastatic tumor cells. Killeen and coworkers [64] recently studied the role of endotoxin and TLR4 in invasion of extracellular matrix (ECM) and have shown that endotoxin promotes tumor cell ECM adhesion and invasion through activation of the urokinase plasminogen activator system (a serine protease that turns plasminogen into enzymically active plasmin responsible for blood clot degradation).

It is undisputed that the presence or absence of TLR4 expression on tumor (as well as nontumor) cells can influence different stages of carcinogenesis. Although many reports show clear correlation between chronic microbial infection and cancer initiation (e.g., *H. pylori* infection), others fail to provide evidence of the presence of endotoxin or other TLR4 ligands at cancer initiation sites. An important role is therefore attributed to different molecules of host origin that have lately arisen as potential endogenous ligands of TLR4. These proposed endogenous molecules include different components of the extracellular matrix, intracellular proteins, or modified lipids or lipoproteins (summarized in Table 4). Interestingly, many of them are proposed to activate both TLR4 and TLR2 without having any substantial structural similarity to their natural ligands (endotoxin or lipopeptides, resp.).

Because many (if not most) of the studies describing putative endogenous TLR4 ligands (Table 4) used recombinant proteins and/or commercial reagents with undetermined levels of residual endotoxin, it is reasonable to raise concerns about the purity of the putative ligands used in experiments. The most common methods used to exclude potential endotoxin contamination are the limulus amebocyte lysate (LAL) test and endotoxin neutralization with polymyxin B (PMB). Some researchers demonstrate that their proposed TLR ligands lose their activating capacity after exposure to elevated temperatures. But as described in an excellent review by Erridge [104], these methods have a major shortfall when used in studies describing novel endogenous TLR4 ligands. LAL test, for example, is unable to detect endotoxin in the presence of endotoxin-binding molecules. Furthermore, molecules that bind endotoxin can also prevent its inactivation by PMB. As for the heat

Table 4

Proposed endogenous TLR4 ligand		Reference
Advanced glycation end product low-density lipoprotein	AGE-LDL	[65]
Angiotensin II		[66, 67]
Beta defensin		[68, 69]
Biglycan		[70, 71]
Calprotectin		[72]
Ceramide		[73]
Fibrinogen		[74, 75]
Fibronectin extra domain A	F-EDA	[76, 77]
High-mobility group box 1	HMGB1	[78–81]
Heat shock protein	HSP	[82–85]
Heparan sulfate		[86]
Hyaluronan		[87–92]
Minimally modified (oxidized) low-density lipoprotein	mmLDL	[93–95]
Myeloid-related protein-8/14	MRP-8/14	[96]
Oxidized Palmitoyl-arachidonoyl-phosphatidylcholine	OxPAPC	[97, 98]
Pancreatic adenocarcinoma upregulated factor	PAUF	[99]
Serum amyloid A		[100]
Saturated fatty acid	SFA	[101]
Surfactant protein A		[102]
Tenascin-C		[103]

sensitivity, the biological activity of endotoxin can be greatly reduced by elevated temperatures.

High-mobility group box-1 protein (HMGB1) is a putative TLR4 ligand implicated in cancer. HMGB1 is a nuclear DNA-binding protein that is actively secreted from cells following cytokine stimulation or passively released during cell death. It signals through the receptor for advanced glycation end products (RAGE) [105] and has been implicated in a variety of immune processes and pathological conditions including cancer [106, 107]. In the past few years many studies reported signalization of HMGB1 through TLR4 and declared HMGB1 an endogenous ligand of TLR4 [79, 80, 107]. HMGB1 is connected in several ways to tumor progression and metastasis [105]. On the other hand, HMGB1 released from irradiated or doxorubicin-/oxaliplatin- treated cells can improve immunogenicity of dying tumor cells and therefore help improve tumor antigen presentation [107]. A substantial number of studies show that HMGB1 however binds agonists of TLR, predominantly anionic molecules such as LPS [108], poly(IC), and CpG ODN that activate TLR4, TLR3, and TLR9; therefore, it may act as a chaperone [109, 110], similar to CD14, which stimulates activation of TLR4, TLR3, TLR7, and TLR9 by their agonists [111–113]. Additionally HMGB1 produced in mammalian cell cultures and therefore devoid of bacterial contaminants or endogenous danger signals does not activate TLR4 (unpublished observation). It should therefore be reconsidered whether these TLR4 ligands are not in fact just endotoxin-binding or endotoxin-sensitizing molecules without the intrinsic capability of binding and activating TLR4 on their own [104].

It is difficult to comprehend the multitude of the proposed TLR4 agonists that bear no structural similarity to the lipid A moiety of the LPS that is the only TLR4 agonist that has been prepared by chemical synthesis and whose molecular mechanism of activation is known [15, 16]. With respect to the plausible molecular mechanism of the direct activation of TLR4/MD-2 signaling complex oxidatively modified endogenous lipids seem to be the most likely ubiquitous endogenous agonists (Manček-Keber, manuscript in preparation).

6. Breaking the Immune Tolerance of Tumors by TLR4 Stimulation

Toll-like receptor activation is the trigger that sets the immune system into action. The application of TLR ligands in cancer therapy is therefore an attractive possibility that has been intensively studied in the past years in the context of cancer treatment or prevention (as anti-tumor vaccine adjuvants). Macrophages stimulated by endotoxin respond by secretion of chemokines and proinflammatory cytokines, including TNFα and interleukin-1β, which coordinate local and systemic inflammatory responses [17]. Dendritic cells, stimulated by endotoxin, secrete IL-12, which is important in anti-tumor immunity [114]. Furthermore, TLR4 stimulation induces DC maturation and antigen presentation, which has important effect on adaptive immune responses [4]. TLR stimulation influences antigen processing and presentation [115] by affecting the expression of costimulatory molecules on the surface of antigen-presenting cells as well as by controlling antigen uptake [116, 117] and phagosome maturation [118]. In addition to presenting antigens to lymphocytes, mature DCs are also capable of activating cancer-specific natural killer and NKT cells [119]. Inversely, TLR-stimulated NK cells facilitate in immature DC activation and maturation [120] and help intensify DC-mediated antitumor immune responses [121].

Tumors consist in large part not only of tumor but also of immune cells. It is therefore reasonable to assume that direct application of TLR ligands will affect both types of cells. TLR stimulation will possibly have even greater effect on the immune cell population, since not all tumor cells express TLR or the expression varies depending on the developmental stage of the tumor.

This is evident form an example of Bacillus Calmette-Guerin (BCG), an attenuated strain of *Mycobacterium bovis* that is used in the current treatment of nonmuscle invasive

bladder cancer [122]. BCG promotes dendritic cell maturation, and this effect is TLR4 (as well as TLR2) dependent [123]. Furthermore, BCG can induce expression of TNF-related apoptosis-inducing ligand (TRAIL) on tumor infiltrating dendritic cells, therefore rendering them cytotoxic against tumor cells [124].

Another example of an immune activator of microbial origin that promotes dendritic cell maturation is the streptococcal agent OK-432. OK-432 is a preparation of a killed low-virulence strain of *Streptococcus pyogenes* that has been successfully used for over 30 years as an immunotherapeutic agent in different malignancies [125]. Its mechanism of action apparently involves TLR4 activation, since OKA-432 does not inhibit tumor growth on TLR4 knockouts as it does on wild-type mice. Moreover, patients with head and neck cancer responded to OK-432 treatment combined with fluoropyrimidine chemotherapy and radiation significantly better if they expressed TLR4 and MD-2 mRNA (compared to patients without TLR4 or MD-2 expression) [126, 127].

Stimulation of TLR4 on tumor cells can give contradicting results in terms of cancer progression versus treatment. The outcome seems to be species, tissue, and tumor type dependent. While TLR4 stimulation is on one hand associated with cancer progression (discussed above), it can also lead to anti-tumor immune response. B16 melanoma cells, for instance, that were stimulated with endotoxin *in vitro* exhibit reduced capability of inducing tumor growth *in vivo*. This response was totally independent of TLR4 expression by nontumor cells. *In vitro* stimulated tumor cells seem to differentially influence the phenotype of tumor infiltrating lymphocytes (TILs) so that TILs produced elevated levels of IFN-gamma and reduced levels of IL-10, thus favorably affecting the intratumoral cytokine balance [10].

7. Radio- and Chemotherapy Can Enhance Antitumor Immunity by Providing TLR Ligands

Combining immunotherapy and radiation is a new, compelling approach to cancer therapy. Though radiation is considered mostly immunosuppressive, it is noted also for its immunostimulatory effects. Patients therefore benefit from radiation therapy not only because it directly damages tumor cells but also because suppressor T-cell populations appear to be more radiosensitive than effector T lymphocytes [128]. Radiation can benefit anti-tumor immunity also by increasing expression of inflammatory cytokines by dendritic cells, therefore affecting their phenotype and function [129]. Dendritic cells are critical for anti-tumor immunity because of their ability to cross-present tumor antigens to specific CD8+ T lymphocytes. For efficient antigen cross-presentation, DCs need to receive appropriate stimulation through innate immune receptors. Since immature DCs can induce anti-tumor immunity when administered into irradiated tumors without the addition of TLR ligands [130], radiation was hypothesized to provide the necessary stimulus.

Apetoh et al. [107] recently proposed that HMGB1, which is released from irradiated tumor cells, acts as an endogenous TLR4 ligand. They demonstrated that TLR4 is essential for efficient tumor antigen cross-presentation following radio- or chemotherapy and proposed that HMGB1 binds and activates TLR4 on DCs. HMGB1 could therefore activate DCs and prevent the accelerated degradation of the phagocytosed tumor antigens within DCs promoting efficient tumor antigen processing and cross-presentation [107] (Figure 2).

The crucial role of TLR4 in immunostimulatory effects of radiation was also emphasized in a study by Paulos et al. [131], where they demonstrated elevated serum levels of endotoxin in mice following whole body irradiation. They showed that microbial endotoxin that translocated from the radiation-injured gut was responsible for enhanced anti-tumor effect of radiation. Moreover, radiation had diminished effect on tumors following removal of translocated endotoxin or in mice that were defective in the TLR4 signaling pathway [131]. These findings could be especially relevant for the treatment of gastrointestinal malignancies.

8. Cancer Vaccines Utilizing TLR4 Activation

Tumor cell lysates or purified tumor-associated antigens for vaccines have been used for therapeutic or prophylactic cancer vaccine. Although cell lysates contain endogenous danger signals that act as adjuvants, strong response against tumor-associated antigens requires additional stimulation of adaptive immune response by Toll-like receptor agonists. Agonists of TLR9 (CpG ODN), TLR3 (poly(IC), and TLR4 (endotoxin analogues) have been used to increase the innate immune response and activate antigen-presenting cells of the host. TLR4 is particularly important for development of a strong adaptive immune response by stimulation of the antibody class switching, affinity maturation, and formation of memory cells [132]. TLR4 is expressed on follicular dendritic cells that are essential for the affinity maturation in germinal centers [133, 134]. Systemic effect and toxicity of LPS preclude its application for cancer immunotherapy that started by the early attempts by William Coley. MPLA is a monophosphorylated lipid A derivative that has several orders of magnitude lower toxicity than lipid A and was reported to preferentially activate TRIF-dependent pathway [135]. MPLA has been registered as a vaccine adjuvant and used in clinical vaccines, such as Cervarix against human papilomavirus. MPLA is the only TLR4 agonist that has been clinically tested as an adjuvant for cancer vaccines. Results in clinical trials have been modest but seem to be much better if the vaccines are used in early stages of the disease, such as, for example, therapy of non-small-cell lung carcinoma (NSCLC) using MAGE-3 antigen combined with MPLA-based adjuvant AS02B rather than in late stages, when the immune system of patients is already severely compromised (reviewed in [136]). Additional alternative therapeutic approaches are based on combination of TLR4 agonist as a vaccine adjuvant with tumor-associated antigens in combination with radio- or chemotherapy or autologous dendritic cell therapy.

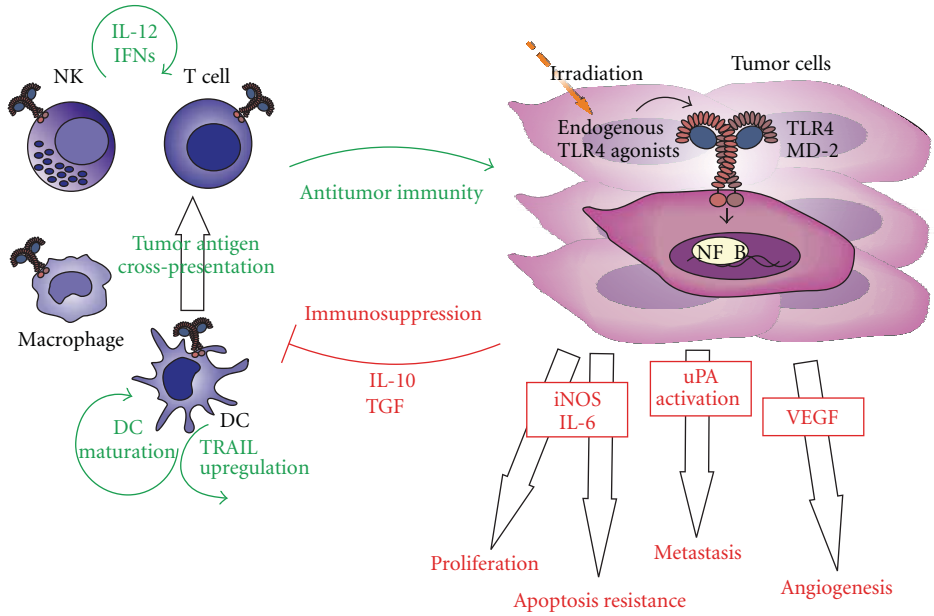

FIGURE 2: TLR4 signaling in cancer—a struggle of antitumor immunity against cancer proliferation and immune evasion. TLR4 signaling on immune cells can enhance anti-tumor immunity by different mechanisms, including IL-12 or IFNγ upregulation and promotion of DC maturation and function (left side of the figure, depicted in green). On the other hand, TLR4 signaling on tumor cells can increase their tumorigenic potential (right side of the figure, depicted in red).

9. Conclusions

TLR signaling triggers immune cell activation and maturation and is indispensable for the efficient immune response against the pathogenic microorganisms as well as against malignant cells. An effective immune system is most important in the early stages of carcinogenesis when cancerous cells are few and are not limited to less immunogenic cell clones. If immunosurveillance against malignantly transformed cells is unsuccessful in the early stage, tumors quickly outgrow the immune cell cytotoxic capabilities. TLR4 expression by tumor cells can be a contributing factor that promotes tumor cell proliferation, survival, or immunosuppression.

Therapeutic interventions at the level of TLR4 stimulation is a double-edged sword since different studies demonstrate positive as well as negative effects of TLR4 stimulation on cancer development or treatment. Harnessing the beneficial effects of TLR4 stimulation while eliminating the negative ones remains the challenge for cancer researchers.

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Review Article

T Cells and Adoptive Immunotherapy: Recent Developments and Future Prospects in Gastrointestinal Oncology

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Gastrointestinal oncology is one of the foremost causes of death: the gastric cancer accounts for 10.4% of cancer deaths worldwide, the pancreatic cancer for 6%, and finally, the colorectal cancer for 9% of all cancer-related deaths. For all these gastrointestinal cancers, surgical tumor resection remains the primary curative treatment, but the overall 5-year survival rate remains poor, ranging between 20–25%; the addition of combined modality strategies (pre- or postoperative chemoradiotherapy or perioperative chemotherapy) results in 5-year survival rates of only 30–35%. Therefore, many investigators believe that the potential for making significant progress lies on understanding and exploiting the molecular biology of gastrointestinal tumors to investigate new therapeutic strategies such as specific immunotherapy. In this paper we will focus on recent knowledge concerning the role of T cells and the use of T adoptive immunotherapy in the treatment of gastrointestinal cancers.

1. Introduction

Gastrointestinal oncology is one of the foremost causes of death; regarding the gastric cancer (GC) the American Cancer Society estimated one million new cases, nearly 70% of them in developing countries, and about 800,000 deaths [1]; instead the pancreatic cancer (PC) is the fourth leading cause of cancer deaths among men and women, being responsible for 6% of all cancer-related deaths [2], and finally, the colorectal cancer (CRC) accounted for 9% of all cancer deaths (49, 920) in 2009 [3].

For all these gastrointestinal cancers, surgical tumor resection remains the primary curative treatment but the overall 5-year survival rate remains poor, ranging between 20–25% [4–6]. The addition of combined modality strategies (pre- or postoperative chemoradiotherapy or perioperative chemotherapy) results in 5-year survival rates of only 30–35% [7–9].

Therefore, many investigators believe that the potential for making significant progress lie on understanding and exploiting the molecular biology of gastrointestinal tumors to investigate new therapeutic strategies such as gene therapy [10] and especially specific immunotherapy [11–13].

Evidence from different analysis suggests a key role of the immune system in counterattack of cancer progression: tumors are 100 times more likely to occur in people who take immunosuppressive medications than in people with normal immune function [14], and, in opposition, heightened anti-tumor activity of the immune system has been suggested in many reports of spontaneous cancer regression [15]. Also, a positive correlation between tumor-infiltrating lymphocytes and patients' survival has been observed [16]; moreover tumor-specific T-cell responses have been found in patients with a variety type of tumors [17].

Immune defence against tumor is mediated through antigen-specific and nonspecific immune mechanisms (macrophage and NK cell lineage and soluble factors such as cytokines). The operational, instead, of the antigen-specific immune system is based on a division of tasks between T cells and B cells (Figure 1).

Various reagents (vaccines, infusion of T cells, or cytokines) can stimulate the immune system essentially through

two mechanisms: (1) stimulation of the antitumor response, either by increasing the number of effector cells or by producing soluble mediators (e.g., cytokines); (2) alteration of tumor cells to increase their immunogenicity and susceptibility to immunological defences. However, the cancer cells have developed a number of different strategies to escape immune surveillance such as loss of tumor antigen expression, MHC downregulation, expression of Fas-L that can induce apoptosis in activated T cells, secretion of cytokines such as IL-10 (Interleukin-10) or TGF-β (Tumor grow factor-β), or generation of regulatory T (Treg) cells [18].

The requirement for an immune-based strategy in opposition to cancer is the induction of an effective tumor-specific immunity in order to break immunological tolerance to the tumor and generate antitumor immunity. To achieve this goal, several strategies as in preclinical models as in clinical trials are currently being investigated.

In this paper we will focus on recent knowledge concerning the role of T cells and the use of T adoptive immunotherapy in the treatment of gastrointestinal cancers.

2. Pancreatic Cancer

2.1. In Human and Animal Model T-Cell Response. Over the past 30 years, a large body of data has been accumulated showing that cancer patients generate B and T cells specific to antigens expressed on autologous pancreatic tumor cells [19–25]. PC expresses a variety of cancer-associated antigens that can potentially be recognized by T cells [26, 27]. Recent studies demonstrated that functionally competent $CD4^+$ and $CD8^+$ T cells with specificity for cancer antigens are spontaneously induced in the bone marrow of all PC patients [27, 28]. Moreover, in approximately 50% of the patients, these tumor-specific T cells are also present in the blood. Upon specific stimulation they mainly secrete the type 1 cytokine IFN-γ, which is typical of cytotoxic immune responses. The high incidence of spontaneous T-cell reactivity versus PC is in contrast to observations from numerous other cancer entities that induced cancer-reactive T cells only in 25–60% of the patients [29–31].

T-cell responses are regulated by dendritic cells (DCs), which constantly take up antigens in all tissues and upon in situ activation, stimulate naive T cells. While type I interferons, heat shock proteins, and extracellular matrix degradation products may induce DC activation in cancer tissues, immune-suppressive cytokines (IL-10/TGF-β) inhibit DC activation, and in PC the latter are produced at high concentrations by cancer-induced pancreatic stellate cells, cancer-infiltrating macrophages and mast cells [32], or Tregs [33]. Through recruitment and activation of stroma cell populations, PC generates a predominantly immune-suppressive microenvironment (Figure 2).

The regular induction of T-cell responses in the bone marrow of PC patients is thus intriguing.

The bone marrow is a site of T-cell priming against blood-borne antigens [34]. It can collect soluble cancer antigens released into the blood from necrotic cancer areas.

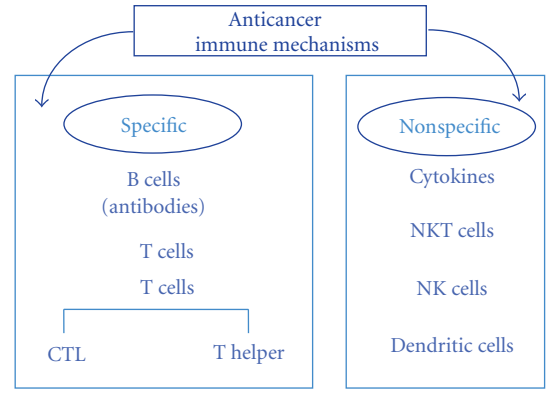

Figure 1: Innate and adaptive immune defence against cancer cells.

Here, these are incorporated and presented by bone marrow-resident DCs in an immune-stimulatory environment. In addition, disseminated neoplastic cells detectable in many patients represent a local source of cancer antigens [28].

PC is frequently diagnosed at late stages. In this situation, large antigen amounts may reach the bone marrow. This might explain the comparably high incidence of T-cell responses in PC despite a predominantly immune-suppressive environment in the primary cancer. Once stimulated, T cells differentiate into effector T cells and enter the blood. Since cancer-reactive T cells have been found in the blood of many PC patients, these cells may infiltrate pancreatic carcinomas.

In one study, cancer-reactive $CD8^+$ T cells specifically lysed autologous PC cells *in vitro* and delayed progression of xenotransplanted, autologous carcinomas [27]. Accordingly, increased numbers of cancer-infiltrating $CD4^+$ and $CD8^+$ T cells correlated well with improved prognosis of PC patients [35].

These findings point to a potential implication of cancer-specific T cells during cancer progression, but PC cells successfully employ various mechanisms to evade immune surveillance (Figure 2): (a) the downregulation of MHC molecules and of fas receptor, rendering neoplastic cells more resistant to recognition and cytolysis by activated effector T cells [27], (b) the recruitment and local maintenance of Tregs [36] that inhibit effector T-cell activation and function, (c) the secretion of IL-10 and TGF-β, additionally reducing local T-cell activity [27, 37], (d) the inactivation of cancer-infiltrating T cells as shown by a severe loss of CD3 zeta, [37] and (e) the expression of fas ligand on neoplastic cells, inducing apoptosis in cancer-infiltrating effector T cells [38].

Thus, PC is not characterized by a lack of specific T-cell immunity but by a potent barrier established by complex cancer-stroma interactions that inhibit T-cell activity in situ; for this purpose is most explanatory the recent results obtained by De Monte et al. [39]; they showed that thymic stromal lymphopoietin (TSLP), which favors Th2 cell polarization through myeloid DC conditioning, was secreted by cancer-associated fibroblasts (CAFs) after activation with tumor-derived TNF-α and IL-1β. Also the authors found that the ratio of GATA-3^+(Th2)/T-bet^+ (Th1) tumor-infiltrating T cells is an independent predictive marker of patient

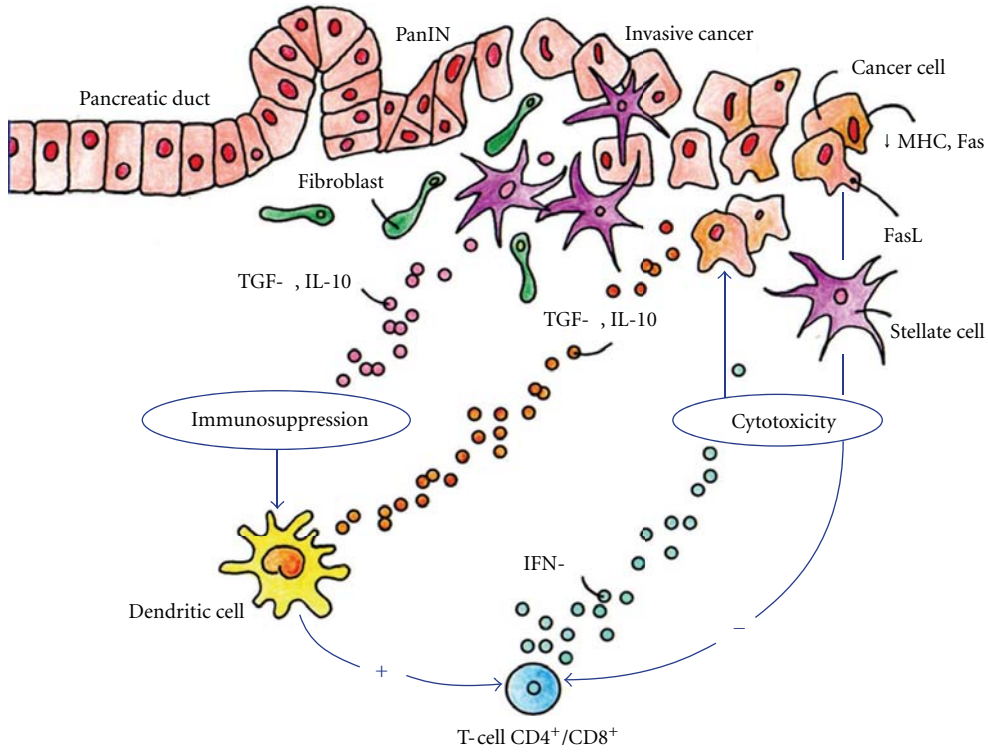

Figure 2: Pancreatic cancer microenvironment: interactions of immune cells with the cancer cells. Yellow: products of stellate cells; green: T-cell derived cytokines; grey: cancer cell-derived factors.

survival. Patients surgically treated for stage IB/III disease with a ratio inferior to the median value had a statistically significant prolonged overall survival, implying an active role for Th2 responses in disease progression.

In addition, in a mouse model in which an activating K-Ras mutation is expressed in the pancreas, preinvasive pancreatic lesions are characterized by the infiltration of immune suppressor cells rather than immune effector cells, suggesting that tumor immunity may be blocked from the inception of PC development [40].

All mice with the K-Ras mutation develop pancreatic adenocarcinoma and eventually die of disease. Finally, the finding that antagonism of negative T-cell regulators, such as cytotoxic T-lymphocyte-associated (CTLA) protein-4 and B- and T-lymphocyte attenuator (BTLA), can augment the antitumor immune response confirms that patients mount an immune-specific response to their tumor [41, 42]. Despite mounting evidence that an antitumor immune response is elicited in cancer patients, this response is ineffective and does not result in the tumor eradication, and a better understanding of the mechanisms underlying these interactions is required to develop future therapeutic strategies to employ the patient's own T-cell arsenal for efficient cancer control.

2.2. T-Cell Immunotherapy of Pancreatic Cancer. The history of vaccine trials in pancreatic cancer targeting a defined PC antigen started with the publication of a pilot study of mutant ras peptide vaccines tailored to represent the K-RAS mutations identified in biopsies from the patients with cancer [43] In this trial, immune responses specific for individual ras mutations were obtained in 2 of the 5 patients enrolled; in addition, both patients had a relatively long survival (11 and 8 months). These data shown that: (a) patients with metastatic PC were immunocompetent, (b) mutant ras vaccines were immunogenic, and (c) immune responses were correlated with survival. Furthermore, the treatment was well tolerated as no adverse effects were observed. A fine evaluation of the immune responses in these two patients [44] highlighted that peptide vaccination with a single mutant p21-ras-derived peptide induced $CD4^+$ and $CD8^+$ specific for nested epitopes, including the Gly/Val substitution at codon 12 and that both these T-cell subsets specifically recognize tumour cells owning to the corresponding K-ras mutation. Encouraged by these results, a second trial was performed, using intradermal vaccination of mutant ras peptides with GMCSF (Granulocyte-macrophage colony-stimulating factor) as an adjuvant [45]. 48 patients (10 surgically resected and 38 with advanced disease) were treated on an outpatient basis. Peptide-specific immunity was induced in 25 of 43 (58%) evaluable patients, indicating that the protocol used is very potent and able to elicit immune responses even in patients with end-stage disease. This study also demonstrated a strong association between immune responses and prolonged survival. Patients with advanced cancer and with immune response to the peptide vaccine showed prolonged survival from the start of treatment compared to nonresponders (median survival 148 days versus 61 days). Furthermore, the study proved long-term memory in numerous patients and entry of vaccine-specific T cells into the tumour mass.

In recent years, much work has focused on adoptive tumor immunotherapy in which the T cells of cancer patient are expanded and reinfused into the patient.

One method results in the selective expansion of T cells endogenously expressing TCRs specific for the tumor antigen of interest [46]. In a clinical study, MUC-1-specific autologous T cells, isolated from patient PBMCs (peripheral blood mononuclear cells), were expanded by incubation with a MUC-1-presenting cell line prior to administration to PC patients. The mean survival time for unresectable patients in this study was 5 months [47]. However, patients with resectable pancreatic cancer had 1-, 2- and 3-year survival rates of 83.3, 32.4, and 19.4%, respectively, and a mean survival time of 17.8 months. In a similar study, the same group isolated adherent cells from patient PBMCs to generate mature DCs that were then pulsed with MUC-1 peptide. The pulsed DCs were administered, along with autologous expanded MUC-1-specific T cells, to patients with unresectable or recurrent pancreatic cancer. Remarkably, a complete response was observed in one patient with lung metastases, and the mean survival time of the whole group was 9.8 months, suggesting that the addition of pulsed DCs may have improved the outcome [48].

A key role in future immunotherapeutic treatment of PC patients seems to be for the novel antigen PC-associated α-enolase (ENOA), a metabolic enzyme involved in the synthesis of pyruvate. In tumor cells, ENOA is upregulated and supports anaerobic proliferation (Warburg effect); also, it is expressed at the cell surface, where it promotes cancer invasion. ENOA is upregulated in different tumors, including brain, breast, cervix, colon, gastric, kidney, lung ovary, and especially pancreas [49].

In pancreatic cancer, ENOA elicits a $CD4^+$ and $CD8^+$ T-cell response both *in vitro* and *in vivo* [49]. Anti-MHC class I antibodies inhibited the cytotoxic activity of ENOA-stimulated $CD8^+$ T lymphocytes against PC cells, but no MHC class I restricted peptide of ENOA has been identified so far. Moreover, in pancreatic ductal adenocarcinoma patients, production of anti-ENOA Immunoglobulin-G (IgG) is correlated with the ability of T cells to be activated in response to the protein [49], thus confirming the induction of a T- and B-cell integrated antitumor activation against ENOA. In oral squamous cell carcinoma, an HLA-DR8-restricted peptide (amino acid residues 321–336) of human ENOA recognized by $CD4^+$ T cells and able to confer cytotoxic susceptibility has been identified [50, 51].

Most importantly, clinical correlations [52–54] propose ENOA as a novel target for cancer immunotherapy, in particular in pancreatic cancer, where pancreas-specific Ser 419 phosphorylated ENOA is upregulated and also induces the production of autoantibodies with diagnostic and prognostic value [49].

3. Gastric Cancer

3.1. Gastric Cancer-Infiltrating T Cells . Although the GC etiology has been completely obscure for many decades, several considerable advances in the knowledge of the carcinogenesis and development of gastric cancer have been made in the present era. First, it is well known that *Helicobacter pylori* (*H. pylori*) infection is associated with the GC carcinogenesis, suggesting that chronic inflammation may be implicated in the development of intestinal metaplasia and mutations in oncogenes that precede the GC development; indeed, the International Agency for Research on Cancer classified *H. pylori* as a class I human carcinogen in 1994 [55]. Second, the long-suspected influence of genetic susceptibility has been elucidated, and several polymorphisms of inflammatory cytokine genes have been implicated as risk factors for gastric cancer [56–60].

Although immune cells constitute an additional and prominent component of the host response to cancer, their participation in tumor pathogenesis remains unclear. In the tumor microenvironment, there is a delicate balance between antitumor immunity and tumor-originated proinflammatory activity, which weakens antitumor immunity [61–63].

It has been shown that the infiltrating grade of $CD3^+$ tumor-infiltrating lymphocytes (TILs) was correlated with a favorable outcome in patients with several types of cancer, including gastric cancer [64]. Thus, it is imperative to understand immunoregulation in gastric cancer, in order to develop novel treatment strategies or improve the efficacy of standard therapies.

The first evidence of correlation between T-cell response and GC was the study of Ren et al. [65] that reported a shift from Th1 to Th2 pattern of cytokine secretion in gastric cancer and has suggested that this may be a critical factor in promoting growth of neoplastic cells. However, our data [66] of tumor-infiltrating and perilesional *H. pylori*-specific T cells failed to confirm such a Th1-Th2 shift. Rather, the major difference between the gastric T-cell clones from uncomplicated chronic gastritis and those from gastric cancer was the degree of expression of cytolytic activity. Indeed, in all patients studied, virtually all the *H. pylori*-specific $CD4^+$ clones derived from gastric tumors or perilesional mucosa consistently expressed perforin-mediated cytolytic potential and Fas-Fas ligand-mediated proapoptotic activity against target cells.

Most recently, Maruyama et al. [67] investigated the distribution of Th17 (T helper 17) cells in relation to Treg as in the TILs as in peripheral blood of GC patients. They showed that in TILs from patients with early disease, the frequency of Th17 cells was significantly higher than that in the normal gastric mucosa (23.7 ± 8.9 versus $4.5 \pm 3.1\%$). Besides, in TILs from patients with advanced disease ($n = 28$), the frequency of Th17 cells was also significantly higher, but lower compared to early disease, than that in the normal gastric mucosa (15.1 ± 6.2 versus $4.0 \pm 2.0\%$). When the ratio of Th17/Treg in TILs was evaluated in individual cases, it was more markedly increased in early than in advanced disease.

In summary, the accumulation of Th17 cells as well as Tregs in the tumor microenvironment of gastric cancer occurred in early disease, and then the infiltration of Th17 cells gradually decreased according to the disease progression, in contrast to increased Tregs.

3.2. T-Cell-Based Antigastric Cancer Treatments. There are different types of T-cell-based anticancer therapy approaches, using (a) CTL, (b) TILs, or (c) Engineered T cells.

Improved CTL cell culture technology has permitted the first clinical tests for adoptive transfer of CTLs, and this technique [68] seems to result in substantial activity in patients with melanoma; CTLs were used to treat patients with metastatic melanoma, and 8 out of 20 patients had antitumor immune responses [68]. These results were confirmed in an independent trial in which engraftment of the CTLs, as measured by an elevated frequency of circulating T cells able to bind tetramers loaded with MART-1 peptides, was detectable up to two weeks after T-cell transfer in all patients [69].

Recently, Kim et al. [70] evaluated the antitumor activity of *ex vivo* expanded T cells against the human gastric cancer. For this purpose, human peripheral blood mononuclear cells were cultured with IL-2-containing medium in anti-CD3 antibody-coated flasks for 5 days, followed by incubation in IL-2-containing medium for 9 days. The resulting populations were mostly $CD3^+$ T cells [97%] and comprised 1% $CD3^-$ $CD56^+$, 36% $CD3^+CD56^+$, 11% $CD4^+$, and 80% $CD8^+$. This heterogeneous cell population was also called cytokine-induced killer (CIK) cells. CIK cells strongly produced IFN-γ, moderately TNF-α, but not IL-2 and IL-4. At an effector-target cell ratio of 30 : 1, CIK cells destroyed 58% of MKN74 human gastric cancer cells, as measured by the 51Cr-release assay. In addition, CIK cells at doses of 3 and 10 million cells per mouse inhibited 58% and 78% of MKN74 tumor growth in nude mouse xenograft assays, respectively. This study suggests that CIK cells may be used as an adoptive immunotherapy for GC patients.

The adoptive GC immunotherapy with CIK cells has been also reported in preclinical and clinical studies [71]. MHC-I restricted CTLs from GC patients recognize tumor-associated antigen and react specifically against self-tumor cells [72, 73]. One tumor-specific antigen, MG7-antigen, shows great potential for predicting early cancer as well as for inducing immune responses to GC [74, 75]. Using HLA-A-matched allogeneic gastric cancer cells to induce tumor-specific CTLs appears to be an alternative immunotherapy option for gastric cancer [76].

Also, CIK cells in combination with chemotherapy showed benefits for patients who suffer from advanced gastric cancers [77, 78]. The serum levels of the tumor markers were significantly decreased, the host immune function was increased, and the short-term curative effect, as well as the quality of life, was improved in patients treated by chemotherapy plus CIK cells compared to those in patients treated by chemotherapy alone. CIK cells killed MGC-803 GC cells by inducing apoptosis in the early stage and by inducing necrosis in the late stage through downregulation of p53, c-myc, and bcl-2 and upregulation of bax [79].

In summary, despite the introduction of immune cell-based immunotherapy, the paucity of preclinical and clinical studies has limited the broad application of immunotherapy for the treatment of GC patients with gastric cancers. Here, preclinical evidence proved that CIK cell immunotherapy can be used in patients with gastric cancer.

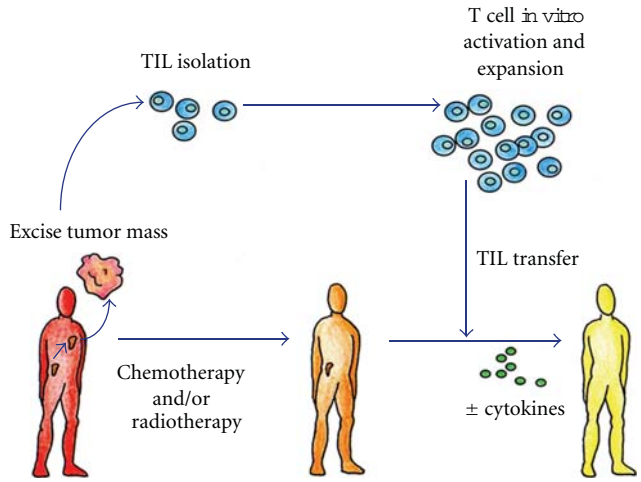

Figure 3: Scheme of adoptive autologous TILs transfer. T-infiltrating lymphocytes can be isolated from resected surgical samples and expanded *in vitro* for adoptive transfer after lymphodepleting chemotherapy. Most adoptive transfer therapy approaches using TILs have involved the use of IL-2 infusion following T-cell transfer in order to select tumor-specific T cells.

Adoptive transfer therapy with TILs requires the isolation of T cells from neoplastic biopsies or surgical tissue and the selection of tumor-specific T cells *ex vivo* (Figure 3). The adoptive transfer of TILs has been promising in preclinical models [80], but clinical experiences were almost uniformly disappointing [81, 82].

Technical difficulties in producing tumor-specific T cells currently represent a barrier to randomized clinical trials. Only 30%–40% of the biopsies yield satisfactory T-cell populations, and the whole process requires about 6 weeks before the T cells would be ready for infusion [83]. Furthermore, nearly all clinical experiences with TILs have been done in patients with melanoma, because of the easy surgical availability of the tumor tissue. However, should technical limitations of current tissue culture approaches be overcome, recent studies indicate that the presence of TILs positively correlates with patients survival in ovarian and colorectal cancer [84, 85], thus prompting the use of this protocol for other commonly encountered epithelial neoplasias. Recently we have [11] analyzed the functional properties of the T-cell response to different antigen peptides related to GC in patients with gastric adenocarcinoma. To this purpose, we have cloned and characterized TILs isolated from the neoplastic gastric tissue samples. A T-cell response specific to different peptides of gastric cancer antigens tested was documented in 17 out of 20 patients, selected for their HLA-A02 and/or -A24 alleles. Most of the cancer peptide-specific TILs expressed a Th1 profile and cytotoxic activity against target cells. The effector functions of cancer peptide-specific T-cells obtained from the peripheral blood of the same patients were also studied, and the majority of peripheral blood peptide-specific T cells also expressed the Th1 functional profile.

In conclusion, in most of patients with gastric adenocarcinoma, a specific type-1 T-cell response to GC antigens was

detectable and would have the potential of hamper tumor cell growth. However, in order to get tumor cell killing *in vivo*, the activity and the number of cancer peptide-specific Th1 cells probably need to be enhanced by vaccination with the appropriate cancer antigenic peptides or by injection of the autologous tumor peptide-specific T cells expanded *in vitro*. These studies have laid the groundwork for a possible vaccination of gastric adenocarcinoma patients with specific peptides of tumor-associated antigens able to raise an effective immune response to gastric cancer.

4. Colorectal Cancer

4.1. Tumour-Infiltrating T-Cell Subsets in Colorectal Cancer. In recent years, different studies demonstrated the presence of T cell into neoplastic tissue of colorectal patients and also that the type, location, and density of tumor-infiltrating immune cells are of strong predictive impact influencing the behavior of human CRC [85, 86]. Although the exact mechanism remains uncertain, the adaptive immune system plays an important role in suppressing tumour progression [87, 88]. In the Table 1 we resumed the major studies correlating the TIL subsets and survival of CRC patients.

From the above, the tumour-infiltrating T cells may be at the same time, an indicator of the host immune response versus cancer cells and an attractive target for immunotherapy [18, 89, 90].

The TILs may also reflect specific molecular alterations associated with indolent tumour behaviour. Previous studies have shown that lymphocytic infiltration is associated with microsatellite instability (MSI) in colorectal cancer [91–93]. Truncated peptides produced by frameshift mutations due to MSI may be immunogenic and contribute to the host immune response [88, 89, 94]. However, at the time, very little is known about the interrelationship between TILs, MSI, and other tumour molecular features, such as the CpG island methylator phenotype (CIMP), global DNA hypomethylation, and KRAS, BRAF, and PIK3CA mutations.

Previous studies have reported that MSI [95], CIMP [96], BRAF mutation [97], *PIK3CA* mutation [98], and tumour LINE-1 hypomethylation [99] are associated with prognosis and that lymphocytic infiltration is associated with many of these molecular variables [92]. As such, to define the prognostic effect of tumour-infiltrating T cells independently of those potential confounders, large studies of colorectal cancers with extensive molecular characterization are needed. Most recently, Nosho and coll. [100], using a database of 768 colorectal cancers, analyzed the subsets of TILs in relation with molecular changes in patients with CRC. They demonstrated that the densities of $CD8^+$, $CD45RO^+$, and $FOXP3^+$ cells were significantly associated with patient survival in univariate analyses (P trend < 0.007). In the multivariate model, tumour-infiltrating $CD45RO^+$-cell density, but not $CD3^+$, $CD8^+$, or $FOXP3^+$-cell density, was significantly associated with survival ($P = 0.0032$). In multivariate linear regression analysis, MSI-high ($P < 0.0001$) and high-level tumour LINE-1 methylation ($P = 0.0013$) were independently associated with higher $CD45RO^+$-cell density. The survival benefit associated with $CD45RO^+$ cells was independent of MSI and LINE-1 status. In conclusion, tumour-infiltrating $CD45RO^+$-cell density is a prognostic biomarker associated with longer survival of colorectal cancer patients, independent of clinical, pathological, and molecular features. In addition, MSI-high and tumour LINE-1 methylation level are independent predictors of $CD45RO^+$-cell density. These results offer a possible mechanism by which MSI confers an improved clinical outcome and support efforts to augment the host immune response in the cancer microenvironment as a strategy of targeted immunotherapy.

As with all tumors analyzed so far, even for the CRC it is very important to evaluate the impact of Tregs on the specific immune responses against tumor-associated antigens (TAAs). The grade of local infiltration did not correlate with responses against well-defined TAAs as EpCAM, Her-2/neu, and CEA [101]. Depleting Tregs in PBMCs from CRC patients dramatically boosted the IFN-γ and TNF-α production in T cells, which were stimulated with a CEA peptide [102]. In spite of the unmasking of responses in opposition to other TAAs, recall antigens such as PPD were not affected suggesting a TAA-specific rather than a systemic immune suppression [103].

In an extremely ample analysis various TAA-specific Tregs were exclusively identified in CRC patients. Peptides for CEA, telomerase, Her-2/neu, and MUC-1 all led to an activation of Tregs [104]. TAA-specific Tregs were successfully identified using a p53 peptide [105]; in addition to $CD4^+$ Tregs also $CD8^+CD28^-$ Tregs could be isolated from peripheral blood, tumor tissue, and metastatic lymph nodes of CRC patients [106]. These cells suppressed T cells in an IL-10-dependent fashion and were mainly $CD194^+$, which may have contributed to their accumulation *via* recruitment. A recent study identified circulating and tumor-infiltrating $CD28^+CD8^+$ Tregs with a $CD25^+$, $FOXP3^+$, $CD152^+$, $GITR^+$, $CD194^+$, TGF-β^+, and $CD127^-$ phenotype [107]. Remarkably this type of Tregs was found in 90% of the CRC specimens but was totally absent in normal colonic tissue suggesting a cancer-specific presence without contribution to the physiologic epithelial homeostasis [108]. Ligands for CD194 (e.g., CCL17 or CCL22) were in contrast to IL-6 and TGF-β not highly expressed in the tumor tissue, altogether indicating a conversion from $CD8^+$ rather than a tumor-directed migration as the cause for the observed infiltration. In another recent study CXCL11 produced by CRC-derived $CD68^+$ myeloid cells is suggested to be a promising chemoattractant for Tregs [109].

4.2. T-Cell-Based Immunotherapy in CRC Patients. T-cell-based immunotherapy (TCI) was first described in 1988 [110], but the decisive improvement in efficacy came in 2002 with the introduction of an immunodepleting preparative regimen given before the adoptive transfer, which could result in the clonal repopulation of patients with antitumour T cells [111]. Of patients with metastatic melanoma refractory to all other treatments, 50% will experience an objective response, some with complete responses [112]. Responses can be durable and are seen in all organ sites, including the brain. Recent studies demonstrating that normal human

Table 1: Studies correlating colorectal cancer patient survival with TIL subsets.

N° of events				High density of T-cells (versus low density)				References
Sample size	OS	CS	Disease stage	T-cell subset analysed	5-year CS, OS, or DFS, long-rank P value	CS, OS, or DFS univariate HR (95% Cl)*, P value	CS, OS, or DFS multivariate HR (95% Cl)*, P value	
131	—	—	Dukes' A–D	$CD8^+$	$P = 0.0003$ (OS)	—	0.61 (0.41–0,89)	[139]; 58: 3491–3494
109	37	—	II-III	$CD3^+$	$P = 0.004$ (OS)	—	$P = 0.011$ (OS) 0.40 (0.19–0.85) (OS)	[140]; 159: 297–304
				$CD8^+$	$P = 0.0008$ (OS)	—	0.33 (0.15–0.73) (OS)	
				$GZMB^+$	$P = 0.0001$ (OS)	—	0.23 (0.10–0.50) (OS)	
93	59	47	Dukes' C	$CD8^+$	77% (versus 38%) $P = 0.011$ (CS)	—	—	[141]; 17: 25–29
				$CD45RO^+$	66% (versus 33%) $P = 0.002$ (CS)	—	—	
				$CD68^+$	60% (versus 37%) $P = 0.033$ (CS)	—	—	
72	—	—	I–IV	$CD134^+$	$P = 0.02$ (CS)	—	—	[142]; 183: 512–518
41	25	—	Dukes' A–D	$CD4^+/CD8^+$ ratio	22% (versus 61%) (OS)	—	$P = 0.046$ (OS)	[143]; 52: 423–428
97	—	—	—	$CD8^+$	—	$P = 0.01$ (OS)	—	[144]; 10: 309–313.
152	—	—	III	$CD8^+$	$P < 0.001$ (CS)	—	—	[145]; 35:808–816
93	—	—	II-III	$CD8^+$	$P = 0.03$ (DFS)	—	0.56 (0.32–0.99) $P = 0.04$ (DFS)	[146]; 84: 493–501
371	—	74	I–IV	$CD8^+$	$P < 0.0001$ (CS)	—	0.43 (0.23–0.83) $P = 0.01$ (CS)	[147]; 91: 1711–1717
336	158	—	Dukes' A–D	$CD45RO^+$	65% (versus 35%) $P < 0.0001$ (OS) 72% (versus 50%) $P < 0.001$ (DFS)	—	$P = 0.02$ (OS)	[148]; 353: 2654–2666

Table 1: Continued.

Sample size	N° of events		Disease stage	High density of T-cells (versus low density)				References
	OS	CS		T-cell subset analysed	5-year CS, OS, or DFS, long-rank *P* value	CS, OS, or DFS univariate HR (95% Cl)*, *P* value	CS, OS, or DFS multivariate HR (95% Cl)*, *P* value	
117	—	—	Dukes' A–D	$CD3^+$	$P < 0.05$ (CS)	—	—	[149]; 4: 1351–1357
				$CD8^+$	$P < 0.25$ (CS)	—	—	
				$CD16^+$	$P < 0.04$ (CS)	—	—	
406	—	—	I–III	$CD3^+$	73% (versus 40%) $P < 0.0001$ (OS) 81% (versus 54%) $P = 0.0012$ (DFS)	—	0.53 (0.40–0.70) $P < 0.0001$ (OS) 0.42 (0.29–0.60) $P < 0.0001$ (DFS)	[150]; 313: 1960–1964
				$CD8^+$	72% (versus 50%) $P < 0.0001$ (OS)	—	—	
				$CD45RO^+$	82% (versus 56%) $P = 0.0002$ (DFS) 68% (versus 33%) $P < 0.0003$ (OS)	—	—	
				$GZMB^+$	77% (versus 37%) $P = 0.002$ (DFS) 72% (versus 61%) $P = 0.15$ (OS)	—	—	
				$CD3^+$, $CD45RO^+$	84% (versus 68%) $P = 0.39$ (DFS) $P < 0.0001$ (DFS)	—	—	
587	—	—	I-II (MSS only)	$CD8^+$	—	0.47 (0.33 0.68) $P < 0.001$ (CS)	0.47 (0.30–0.73) $P = 0.001$ (CS)	[151]; 112: 495–502
392	—	226	I-II (Rectum only)	$CD8^+$	—	0.55 (0.41–0.74) $P < 0.001$ (CS)	0.63 (0.45–0.88) $P = 0.006$ (CS)	[152]; 99: 1712–1717
101	—	—	II-III	$CD3^+/FOXP3^+$ ratio	71% (versus 62%) (OS) 67% (versus 46%) (DFS)	0.57 (0.30–1.09) $P = 0.087$ (OS) 0.46 (0.24–0.90) $P = 0.021$ (DFS)	0.47 (0.24–0.94) $P = 0.039$ (DFS)	[153]; 137: 1270–1279

Table 1: Continued.

Sample size	N° of events		Disease stage	T-cell subset analysed	High density of T-cells (versus low density)			References
	OS	CS			5-year CS, OS, or DFS, long-rank *P* value	CS, OS, or DFS univariate HR (95% Cl)*, *P* value	CS, OS, or DFS multivariate HR (95% Cl)*, *P* value	
286	—	136	II-III	$CD3^+$	Node-negative $P = 0.01$ (CS) Node-positive $P = 0.66$ (CS) Node-negative $P = 0.006$ (DFS) Node-positive $P = 0.62$ (DFS)	—	—	[154]; 10: 877–884
1232	—	—	I-III	$FOXP3^+$	MSS group 162% (versus 46%) $P = 0.004$ (CS)	—	0.73 (0.60–0.90) $P = 0.019$ (CS)	[155]; 126: 2635–2643
					MSS group 2 (CS) 60% (versus 44%) $P = 0.001$ (CS)	—	0.70 (0.60–0.90) $P = 0.007$ (CS)	
					MSI group (CS) 75%? (versus 63%?) $P = 0.029$ (CS)	—	0.63 (0.3–1.2) $P = 0.13$ (CS)	
445	—	—	II-III	$CD8^+$	—	0.74 (0.67–0.82) $P < 0.0001$ (CS)	NS (CS)	[156]; 27: 186–192
				$CD45RO^+$	—	0.74 (0.65–0.84) $P < 0.0001$ (CS)	NS (CS)	
				$FOXP3^+$	—	0.78 (0.70–0.87) $P < 0.0001$ (CS)	0.54 (0.38–0.77) $P = 0.001$ (CS)	
411	—	—	I-II	$CD8^+$	$P < 0.0001$ (OS and DFS)	—	—	[157]; 27: 5944–5951
				$CD45RO^+$	$P < 0.0001$ (OS and DFS)	—	—	
				$CD8^+$ plus $CD45RO^+$	$P < 0.0001$ (OS and DFS)	—	$P < 0.0001$ (CS, OS and DFS)	

Table 1: Continued.

Sample size	N° of events		Disease stage	T-cell subset analysed	High density of T-cells (versus low density)			References
	OS	CS			5-year CS, OS, or DFS, long-rank *P* value	CS, OS, or DFS univariate HR (95% Cl)*, *P* value	CS, OS, or DFS multivariate HR (95% Cl)*, *P* value	
209	100	100	I–IV	$CD3^+$	$P = 0.04$ (OS)	—	0.54 (0.18–1.59) $P = 0.26$ (OS)	[158]; 11: 19
				$CD8^+$	$P = 0.04$ (OS)	—	2.06 (0.67–6.39) $P = 0.21$ (OS)	
				$GZMB^+$	—	—	1.18 (0.45–3.13) $P = 0.74$ (OS)	
94	—	—	I–IV	$CD8^+/FOXP3^+$ ratio	$P = 0.01$ (OS)	0.35 (0.15–0.81) $P = 0.014$ (OS)	0.40 (0.17–0.94) $P = 0.035$ (OS)	[159]; 59: 653–661
57	—	—	—	$FOXP3^+$	—	$P = 0.0009$ (DFS)$P = 0.0005$ (OS)	—	[160]; 33: 435–441
87	—	—	II	$CD3^+$	$P = 0.010$ (DFS) $P = 0.061$ (OS)	$P = 0.003$ (DFS) $P = 0.039$ (OS)	0.20 (0.02–2.60) $P = 0.22$ (DFS)	[161]; 116:5188–5199
				$CD25^+$	$P = 0.013$ (DFS) $P = 0.15$ (OS)	$P = 0.002$ (DFS) $P = 0.017$ (OS)	0.22 (0.02–2.35) $P = 0.21$ (DFS)	
				$CD45RO^+$	$P = 0.049$ (DFS) $P = 0.16$ (OS)	$P = 0.014$ (DFS) $P = 0.037$ (OS)	0.24 (0.02–1.10) $P = 0.014$ (DFS)	
				$FOXP3^+$	$P = 0.009$ (DFS) $P = 0.027$ (OS)	$P = 0.005$ (DFS) $P = 0.040$ (OS)	0.14 (0.07–0.85) $P = 0.027$ (DFS)	
768	366	229	I-IV	$CD3^+$	79% (versus 75%) Q4 (versus Q1)† $P = 0.19$ (CS)	0.73 (0.49–1.08) Q4 (versus Q1)† $P = 0.070$‡ (CS)	1.30 (0.81–2.07) Q4 (versus Q1)† $P = 0.16$‡ (CS)	[162]; 222: 350–366
				$CD8^+$	78% (versus 66%) Q4 (versus Q1)† $P = 0.026$ (CS)	0.61 (0.42–0.88) Q4 (versus Q1)† $P = 0.007$‡ (CS)	0.81 (0.52–1.27) Q4 (versus Q1)† $P = 0.34$‡ (CS)	
				$CD45RO^+$	83% (versus 68%) Q4 (versus Q1)† $P < 0.0001$ (CS)	0.40 (0.26–0.60) Q4 (versus Q1)† $P < 0.0001$‡ (CS)	0.51 (0.32–0.80) Q4 (versus Q1)† $P = 0.034$‡ (CS)	
				$FOXP3^+$	80% (versus 64%) Q4 (versus Q1)† $P < 0.0001$ (CS)	0.48 (0.32–0.70) Q4 (versus Q1)† $P < 0.0001$‡ (CS)	0.89 (0.59–1.34) Q4 (versus Q1)† $P = 0.034$‡ (CS)	

*HR is based on comparing high versus low score of a given T-cell subset. †Quartile of density (Q1-4, first to fourth quartile). ‡*P* for trend. Cl: confidence interval; CS: colorectal cancer-specific survival; DFS: disease-free survival; HR: hazard ratio; MSI: microsatellite instability; NS: not significant; OS: overall survival.

T cell can be genetically engineered to recognize cancer antigens and mediate cancer regression *in vivo* have opened opportunities for enhancing and extending the TCI approach to patients with a wide variety of cancer types [113]. These studies provide a valuable guide to the immunological principles that form the basis of effective immunotherapy for CRC patients.

Most nonhematopoietic tumors such as CRC express MHC class I molecules, but do not express MHC class II molecules, therefore it is believed that the predominant tumor-specific cell-mediated immune effector mechanism is the killing by CTL. However, the clinical history of the patient with cancer often demonstrates the failure of the immune system to eliminate the tumor [114]. It is now generally accepted that this is mostly due to poor tumor-specific MHC class II-restricted $CD4^+$ T helper generated in tumor-bearing hosts [115–117] and that Th cells are required for priming and clonal expansion of specific CTL following reencounter with antigen [118–121].

Although at clinical level, TCI results are still preliminary [122], nevertheless the importance of including $CD4^+$ together with $CD8^+$ T cells to induce optimal therapeutic effects has been established [112, 123].

For this purpose and to optimize the antitumor immunological arms in terms of specificity and long-lasting memory, vaccination with tumor cells transduced with the AIR-1-encoded CIITA, the MHC class II [MHC-II] gene transactivator [124, 125], has been explored with the idea that CIITA-transfected cells may act as "surrogate APC" for optimal triggering of tumor-specific Th cells and thus facilitate the recognition of TAA presented by tumor cell MHC-II molecules. Indeed, the group of Accola showed that complete rejection and long-lasting antitumor memory could be obtained after vaccination with CIITA-expressing TS/A mammary adenocarcinoma [126–128]; Most recently, the same group [129] demonstrated that CIITA-expressing C51 colon adenocarcinoma cells are rejected in high percentage of mice or strongly reduced in growth. Induction of antitumor immunity depended on the ability of the MHC-II-positive tumor cells to trigger $CD4^+$ T cells, which in turn induce stimulation and maturation of CTL effectors. Importantly, they showed that immune $CD4^+$ Th cells can induce protective antitumor responses in naive mice injected with parental nontransfected tumor cells. Purified $CD4^+$ T cells from C51-CIITA vaccinated and challenged mice were also efficacious in preventing tumor growth of C51 tumor, as 50% of the animals were protected and the remaining 50% displayed a significant growth retardation. Similar results were obtained when immune $CD8^+$ T cells were used in adoptive transfer, even if $CD4^+$ T cells were clearly superior to $CD8^+$ T cells in antitumor protective function. Interestingly, the protective phenotype was associated to both a Th1 and Th2 polarization of the immune effectors.

In conclusion, these results demonstrated that tumor cell modification by CIITA may offer an alternative strategy not only for preventive vaccination but also for the generation of more efficacious TCI for CRC patients.

In recent years it has also become increasingly the cancer stem cell theory [130], the idea that cancers are composed of several types of cells, and that only a small population of cancer cells that can regenerate cancer tissues, much as normal tissue can be regenerated only by a small population of stem-like cells. Recently, cancer stem-like cells and tumor-initiating cells (CSCs/TICs) have been isolated from various types of malignancies, including colon cancer [131–135].

In colon cancer, CSCs/TICs can reinitiate tumors that resemble mother colon cancer tissues morphologically when transplanted into immunodeficient mice [132]. Furthermore, these CSCs/TICs have higher tumorigenic potential than do non-CSCs/TICs, suggesting that they are essential for tumor maintenance and distant metastasis [132].

Previous reports have shown that CSCs/TICs are resistant to a variety of treatments, including chemotherapy and radiotherapy, with varied mechanisms of resistance, including high expression of drug transporters, relative cell cycle quiescence, high levels of DNA repair machinery, and resistance to apoptosis [136].

In recent times Inoda and coll. shown that CTL specific for the tumor-associated antigen CEP55 can efficiently recognize colon CSCs/TICs both *in vitro* and *in vivo*. The authors isolated CSCs/TICs as side population (SP) cells from colon cancer cell lines SW480, HT29, and HCT15. The SP cells expressed high levels of the stem cell markers SOX2, POU5F1, LGR5, and ALDH1A1 and shown resistance to chemotherapeutic agents (irinotecan or etoposide). To evaluate the susceptibility of SP cells to CTLs, they used CTL clone 41, which is specific for the CEP55-derived antigenic peptide Cep55/c10orf3_193 [137, 138]. The SP cells expressed HLA class I and CEP55 at the same level as the main population cells. The SP cells were susceptible to CTL clone 41 at the same level as main population cells. Furthermore, adoptive transfer of CTL clone 41 inhibited tumor growth of SW480 SP cells *in vivo*.

These results suggest that Cep55/c10orf3_193 [137, 138] peptide-based cancer vaccine therapy or adoptive cell transfer of the CTL clone is a possible approach for targeting chemotherapy-resistant colon CSCs/TICs.

5. Conclusion

Despite advances in clinical diagnostics, surgical techniques, and development of new chemo/radiotherapy regimens the prognosis of gastrointestinal oncology remains poor, and the need for new treatment options, such as immunotherapy, is imperative.

Studies of T-cell-based immunotherapy have clearly demonstrated that the administration of highly avid antitumour T cells directed against a defined target can mediate the regression of large, vascularized, metastatic cancers and provide guiding principles as well as encouragement for the further development of adoptive T-cell therapy for cancer patients.

In this paper we have reported the evidence of the key role of T-cell response versus cancer of the digestive system and the results obtained in different clinical trials using T-cell immunotherapy.

We showed that for pancreatic cancer as well as for both gastric and colorectal cancer good results were obtained

in some clinical settings but in order that T-cell-based immunotherapy become a real treatment for gastrointestinal oncology, several problems must be solved.

A major problem with the application of TCI is that it is a highly personalized treatment and does not easily fit into current modes of oncological practice. The treatment is expensive, labour-intensive, and requires high laboratory expertise. In essence, a new reagent needs to be created for each patient, and this patient-specific nature of the treatment makes it difficult to commercialize.

Moreover, currently the major challenge in the field is to conduct randomized clinical trials demonstrating sufficient clinical benefit to justify the logistics and costs of customized cellular therapies. In many clinical trials, patients are enrolled at an advanced cancer stage, and this aspect could determine an unfavourable outcome; thus, it would be very interesting to plan clinical trials in early-stage of cancer because it would be possible that gastrointestinal cancer immunotherapeutic approaches confer a survival advantage when applied earlier during the course of the disease, such as in the adjuvant setting.

However, the big hurdle to make immunotherapy approach successful for gastrointestinal oncology remains the immune evasion strategies set up by the tumor resulting in avoidance of both innate and adaptive immunity.

Investigations during the past few years have provided new insights into the cellular and molecular mechanisms involved in the bidirectional crosstalk between cancer cells and the immune cells. Understanding this functional dialogue and the hierarchical status of different tumor-immune escape stratagems at different stages of tumor development will guide the design of novel therapeutic strategies aiming to demolish the "tumor fortress".

Thus, it will be of particular interest to study the kinetics of the interactions between different inhibitory molecules and endogenous factors that influence the expansion and trafficking of Tregs and tolerogenic DCs within tumor-draining lymph nodes and the tumor surroundings.

On the basis of clinical and experimental evidence, it is reasonable to conclude that successful therapy for gastrointestinal oncology must involve a combination approach, which should involve systemic chemotherapy and transplantation to reduce the burden or to eliminate immune suppressive cells, together with tailor-made immunotherapies customized to each single patient.

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Clinical Study

Upregulated Expression of Indoleamine 2, 3-Dioxygenase in Primary Breast Cancer Correlates with Increase of Infiltrated Regulatory T Cells *In Situ* and Lymph Node Metastasis

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IDO has been reported to induce immunotolerance and promote metastasis in solid malignancy, but the mechanisms involved were not fully understood. In this study, the expression of IDO in primary breast cancer was examined and the correlation between the expression levels of IDO and the densities of $Foxp3^+$ Tregs *in situ* was studied. The IDO stably-expressing CHO cells(IDO/CHO) were generated to evaluate the induction of $Foxp3^+$ Tregs after coculturing with $CD3^+$ T cells *in vitro*. The IDO expression in cancer was higher than that in benign diseases both at RNA and protein levels. The IDO expression was significantly upregulated in tumors of more advanced stages and with more extensive lymph node metastasis, and displayed positive linear correlation with the density of $Foxp3^+$ Tregs. We further demonstrated that $CD4^+CD25^+CD127^-$ Tregs could be amplified by coculturing $CD3^+$ T cells with IDO/CHO cells *in vitro* which displayed increasing Foxp3 expression both at mRNA and protein levels. Our results implied that up-regulation of IDO in primary breast cancer may inhibit local immune surveillance and promote metastasis by favoring development and infiltration of $Foxp3^+$ Tregs in the tumor microenvironment.

1. Introduction

Breast cancer is the most common solid malignancy in women worldwide. A substantial fraction of breast cancer patients develop distant metastases shortly after diagnosis. Metastatic breast cancer is associated with poor prognosis with shorter survival time and refractoriness to therapies. Previous studies have proposed the mechanisms of early metastasis, including overexpression of growth factor receptors and resistant to apoptosis [1, 2], downregulation of adherent molecules during epithelial-mesenchymal transition (EMT) [3–5], degradation of extracellular matrix after activation of matrix metalloproteinases (MMPs) [6, 7], enhanced tumor angiogenesis [8, 9], and inhibition of effective antitumor immunity [10, 11]. Breast cancer cells can evade the immune attack through a variety of complex mechanisms, among which tumor-derived immunosuppression resulting from upregulation of metabolistic enzymes, such as indoleamine 2,3-dioxygenase (IDO), has shown a crucial role in the recent studies [12–15].

IDO is a rate-limiting enzyme in the catabolic process of extrahepatic tryptophan which is an essential amino acid for T-cell proliferation and activation. Deprivation of tryptophan in the microenvironment directly affects the cytotoxicity and cytokine secretion of T cells. In addition, the toxic metabolites generated from tryptophan via the Kynurenine pathway directly induce T-cell apoptosis *in vitro* [16]. It is also reported that IDO may inhibit T-cell immunity by inducing differentiation and maturation of $CD4^+CD25^+$ regulatory T cells (Tregs) [17]. Therefore, IDO has been

implicated in the development of autoimmune diseases, regulation of transplantation immunity, and maintenance of maternal-fetal tolerance [18].

Recent studies demonstrated that the expression level of IDO increased in many types of human tumors, including cancers of the lungs, prostate, pancreas, and cervical carcinoma. Tumor-derived IDO dramatically inhibits local T-cell-dependent antitumor immunity and facilitates tumor metastasis [19, 20]. Our previous studies, along with work from other groups, demonstrated that the proportion of $CD4^+CD25^+$ Treg subset increased in breast cancer patients, with strong correlations with the histological grade and the tumor size [21, 22]. However, it is not clear if the increase of $CD4^+CD25^+$ Tregs *in situ* is correlated with the upregulated expression of IDO in tumor cells. In this study, the expression of IDO at both mRNA and protein levels were examined in 26 cases of primary breast cancer and 10 cases of benign breast diseases. The correlation between IDO expression levels and the densities of $Foxp3^+$ Tregs in the primary tumor tissues (PTs) and tumor-draining lymph nodes (TDLNs), as well as various clinical and pathological indexes of the patients were investigated. Our data indicated that the expression of IDO in breast cancer PTs was higher than that in benign disease tissue, but lower than that in TDLNs. IDO was predominantly expressed in cancer cells and modestly expressed in hyperplastic ductal cells and some myeloid cell-like karyocytes in TDLNs. The expression of IDO in PTs was positively linearly correlated to the density of $Foxp3^+$ Tregs in PTs and TDLNs and was significantly higher in tumors of more advanced stages and with more extensive lymph node metastasis. In order to find out if high level of IDO can induce amplification of $Foxp3^+$ Tregs, we cocultured $CD3^+$ T cells with IDO^+ CHO cells (IDO/CHO) *in vitro*. The proportion and absolute number of $CD4^+CD25^+CD127^-$ Tregs increased after coculturing $CD3^+$ T cells with IDO/CHO for 7 days, along with elevated Foxp3 expression at mRNA and protein levels in the $CD3^+$ T cells. These results suggested that upregulation of IDO in breast cancer cells may lead to increased recruitment of $CD4^+CD25^+$ Tregs into the tumor microenvironment and thus inhibit the local immune surveillance and promote metastasis. Therefore, novel IDO-targeted therapies may provide a new direction for the treatment of breast cancer.

2. Materials and Methods

2.1. Patients. Fresh and paraffin-embedded samples, including primary tumors, TDLNs, and normal adjacent tissues were collected from 26 cases of breast cancer patients who were treated with radical mastectomy for breast cancer at the Department of Breast Oncology of Tianjin Cancer Institute & Hospital from June to December, 2009. All patients included 25 females and 1 male with a median age of 50 (31~70) years old, among whom 21 cases of invasive ductal carcinoma, 2 cases of invasive lobular carcinoma, 1 case of invasive micropapillary carcinoma, 1 case of mucinous carcinoma, and 1 case of secretory carcinomas were diagnosed pathologically based on the 2003 WHO classification of breast tumor. According to the 6th edition of the AJCC Cancer Staging Manual, all patients included 2 cases of stage I, 13 cases of stage IIA, 7 case of stage IIB, 3 cases of stage IIIA, and 1 case of stage IIIC. Other 10 patients with benign breast diseases, including 7 cases of breast fibroadenoma and 3 cases of lobular hyperplasia were enrolled as control. This research project was approved by the Ethics Committee of Tianjin Cancer Institute and Hospital. Written consents were obtained from each patient.

2.2. Immunohistochemistry (IHC). Formaldehyde-fixed, paraffin-embedded PTs and TDLNs samples were sectioned into 4 μm slices and affixed on glass slides. The immunohistochemical staining was performed according to the instruction manuals. Briefly, after being heated for half an hour at 56°C, the samples were deparaffinized in xylene and rehydrated through graded alcohol. Antigens were retrieved by heating in citrate buffer for a total of 20 minutes. Endogenous peroxidase activity was quenched in a bath of methanol and hydrogen peroxide for 30 minutes. All samples were incubated overnight at 4°C with mouse anti-human Foxp3 monoclonal antibody (Clone PCH101, eBioscience, San Diego, Calif, USA) and mouse anti-human IDO monoclonal antibody (Clone 10.1, Chemicon, Temecula, Calif, USA) at concentrations of 1 : 1000 and 1 : 500, respectively. These antibodies were detected by a biotinylated secondary antibody (goat anti-mouse IgG-HRP, sc-2302, Santa Cruz, Calif, USA) labeled with streptavidin-horseradish peroxidase (HRP), with the use of a DAB staining kit (Maixin Biotechnology, Fuzhou, China). For negative control, the primary antibody was substituted with PBS. Positive cells were stained brownish yellow in the cytoplasm (IDO-positive staining) or nucleus (Foxp3-positive staining). Two indicators were used to describe the protein expression of IDO and Foxp genes: staining rate (SR) and staining index (SI). The SRs referred to the percentages of positive samples in all samples. The SIs referred to the percentages of positively stained cells in each sample which were calculated using the following formula: (SI = number of positively stained cells/total number of counted cells $\times$100%). The SI was determined upon the average of at least five high-powered fields (400x magnification). An Olympus BX51 microscope was used for image acquisition and data analysis.

2.3. Establishment of Stable IDO^+ CHO Transfectants. A 1225 kb fragment encoding the entire open reading frame (ORF) of human IDO gene was amplified by RT-PCR method using total RNA isolated from MDA-MB-435s breast cancer cells as template. The PCR product was firstly cloned into the pMD19-T Simple Vector (Takara, Japan) and then subcloned into the pIRES2-EGFP vector (Clontech, MountainView, Calif, USA) to generate a recombinant expression plasmid pIRES2-EGFP-IDO. The CHO cells were transfected with pIRES2-EGFP-IDO using a standard electroporation method (field strength of 350 V/cm, 60 μs, 1 pulse), and IDO^+ CHO transfectants (CHO/IDO) were selected by G418 (1 mg/mL, Invitrogen, Carlsbad, Calif, USA) in RPMI 1640 medium supplemented by 10% FBS (Hyclone, Calif, USA) as described previously [23]. CHO cells transfected with pIRES2-EGFP (CHO/EGFP) were used as negative control.

Table 1: Primers for real-time quantitative RT-PCR.

Gene name	Primer sequences	Product size
IDO	UP: 5′-CATCTGCAAATCGTGACTAAG-3′ DP: 5′-CAGTCGACACATTAACCTTCCTTC-3′	188 bp
Foxp3	UP: 5′-CCCACTTACAGGCACTCCTC-3′ DP: 5′-CTTCTCCTTCTCCAGCACCA-3′	486 bp
β-actin	UP: 5′-TGGCACCCAGCACAATGAA-3′ DP: 5′-CTAAGTCATAGTCCGC CTAGAAGCA-3′	186 bp

Note: UP: upstream primer; DP: downstream primer.

2.4. Coculture of CHO/IDO Cells and CD3$^+$ T Cells. The CD3$^+$ T cells in peripheral blood mononuclear cells (PBMCs) of breast cancer patients were purified using Human Pan T-cell Isolation Kit II (Miltenyi Biotec, Germany) according to the manufacturer's instructions. 1×10^5 CHO/IDO cells and CHO/EGFP cells were seeded in a 24-well plate and cocultured with 2×10^6 purified T cells in complete RPMI 1640 medium supplemented with 10% FBS and 50 U/mL rhIL-2 (PeproTech, USA) at 37°C in a 5% CO_2 incubator. Unstimulated T cells cocultured in complete RPMI 1640 medium supplemented with 10% FBS and 50 U/mL rhIL-2 were used as control. The nonadherent T cells under different treatments were harvested 7 days later for flow cytometry analysis, quantitativee real time RT-PCR, and Western Blot analysis.

2.5. Flow Cytometry Analysis. The proportions and absolute counts of Tregs in T cells cocultured with CHO/IDO or CHO/EGFP cells for 7 days, as well as the control T cells were detected by flow cytometry using FITC labeled anti-human CD25, PE labeled anti-human CD127, and PerCP-Cy5.5 labeled anti-human CD4 (BD Biosciences Pharmingen, San Diego, Calif, USA) in TrueCount tubes (BD Pharmingen, San Diego, Calif). The isotype-matched IgG1 was used as negative control to eliminate nonspecific staining. 1×10^5 cells were incubated with antibodies for 30 min on ice in dark. Then, the cells were washed twice with PBS containing 0.2% BSA, fixed using 1% paraformaldehyde and analyzed using a FACSAria flow cytometry (Becton Dickinson, Mountain View, Calif). At least 50,000 events were acquired for each analysis. All samples were measured at least three times.

2.6. Quantitative Real-Time RT-PCR (qRT-PCR) Assay. The mRNA expression of IDO gene in PTs, TDLNs, and normal adjacent tissues, as well as the mRNA expression of forehead transcription factor 3 (Foxp3) gene in stimulated and unstimulated T cells was analyzed using quantitative real-time RT-PCR. The total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, Calif, USA) and reverse transcribed to cDNA using MMLV reverse transcriptase (Promega, Madison, Wis, USA). The expression levels of target genes were quantified using the SYBR Premix Ex Taq system (Takara Bio, Tokyo, Japan) following the manufacturer's instructions. The primers of IDO, Foxp3, and β-actin were listed in Table 1. The thermal cycling program was listed below: initial denaturalization at 94°C for 5 minutes, 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds for 35 cycles; after the last cycle, 72°C for 10 minutes. The products of PCR reactions were analyzed by agarose gel electrophoresis. The relative amounts of IDO and Foxp3 genes were normalized by β-actin and calculated using the formula: $2^{-\ Ct}$($Ct = Ct_{Foxp3} - Ct_{\beta\text{-actin}}$). All tests were repeated at least four times.

2.7. Western Blot. The protein expression of Foxp3 in T cells cocultured with CHO/IDO or CHO/EGFP cells for 7 days, as well as in the control unstimulated T cells, was analyzed using Western Blot analysis. T cells were washed using PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM NaF and 1 μg/mL of aprotinin and leupeptin, pepstatin) on ice. After centrifugation, soluble cellular protein concentration was determined using Micro BCA Protein Assay Kit (Pierce Biotechnology, Ill, USA). The proteins were separated on SDS-PAGE and transferred to PVDF membranes. The membrane was incubated with rabbit polyclonal anti-Foxp3 antibody (BioLegend, San Diego, Calif, USA) overnight at 4°C. Then, the membrane was incubated with HRP-conjugated mouse secondary antibodies (Zhongshanjinqiao, Beijing, China) for 1 h at room temperature. Bound HRP was detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Ill, USA). The intensity of bands was recorded using the ChemiDoc XRS imaging system and analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, Calif, USA).

2.8. Statistical Analysis. All data were presented as mean ± standard deviation (SD). The statistical analysis was performed using a SPSS 13.0 software package. The one-way single factor analysis of variance (ANOVA) was used for the comparison of the quantitative data, and the chi-square (χ^2) test was used for the comparison of the qualitative data. The Spearman's rank-order test and linear regression analysis were used to assess correlations between IDO$^+$ and Foxp3$^+$ SIs. The survival times were compared using Kaplan-Meier Survival analysis. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. The Expression of IDO in Breast Cancer PTs and TDLNs Was Higher Than That in Benign Diseases at Both RNA and Protein Levels. The expression of IDO in 26 cases of

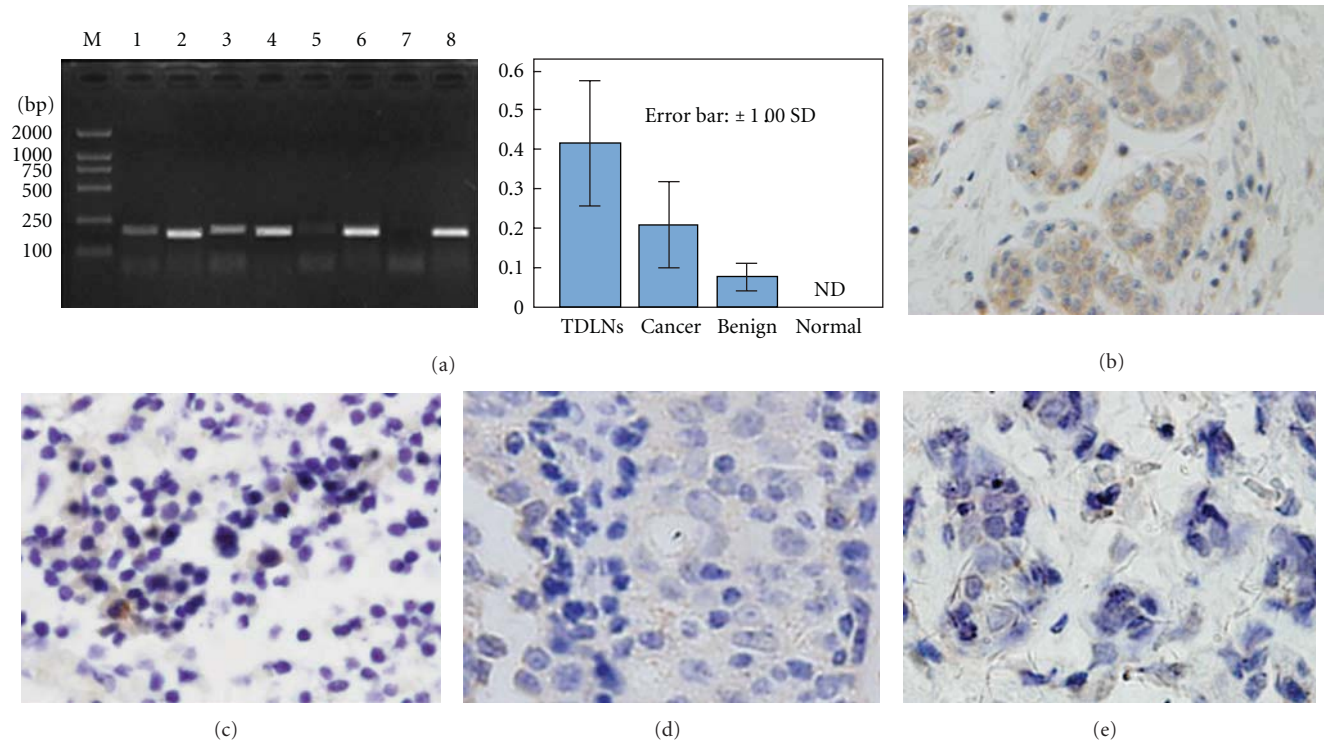

Figure 1: IDO expression in PTs and TDLNs was higher than that in benign diseases at both mRNA and protein levels. The expression of IDO in 26 cases of breast cancer PTs, TDLNs, and normal adjacent tissues and 10 cases of benign breast diseases was detected using qRT-PCR assay and IHC staining method. (a) The IDO mRNA expression in PTs was higher than that in benign diseases but lower than that in TDLNs using the grayscale density ratio of IDO/β-actin (lane 1: IDO in PTs; lane 2: β-actin in PTs; lane 3: IDO in TDLNs; lane 4: β-actin in TDLNs; lane 5: IDO in benign diseases; lane 6: β-actin in benign diseases; lane 7: IDO in normal tissues; lane 8: β-actin in normal tissues; M: DL2000 marker). (b)–(e) The IDO protein was predominantly expressed on cancer cells in breast cancer PTs (b) and in myeloid cell-like karyocytes in TDLNs (c), while less IDO expression was found in mammary ductal cells with hyperplasia in benign diseases (d). No IDO protein expression was detected in normal adjacent tissues (e).

breast cancer PTs, TDLNs, and normal adjacent tissues and 10 cases of benign breast diseases was detected using qRT-PCR and IHC methods. No detectable expression of IDO was observed in the normal adjacent tissues at either RNA or protein level (Figures 1(a) and 1(e)). The IDO mRNA expression in PTs was about 3 times higher than that in benign diseases by comparing the grayscale density ratio of IDO/β-actin (Figure 1(a), $P < 0.05$). Consistently, the IDO$^+$SR and IDO$^+$SI in PTs were significantly higher than that in benign diseases, which were 46.15% (12/26) versus 10.00% (1/10) for IDO$^+$SR and 13.16 ± 7.82% versus 3.24 ± 1.30% for IDO$^+$SI (Figures 1(b) and 1(c), $P < 0.05$). The mRNA expression of IDO in TDLNs was 2 times higher than that in PTs. Accordingly, the IDO$^+$SR and IDO$^+$SI in TDLNs were significantly higher than those in PTs which were 73.08% (19/26) versus 46.15% (12/26) for IDO$^+$SR and 20.46 ± 6.57% versus 13.16 ± 7.82% for IDO$^+$SI (Figures 1(b)–1(d), $P < 0.05$). Furthermore, we found that the IDO$^+$ SIs in TDLNs were significantly positively correlated to those in PTs in a linear pattern as determined using the Regression Analysis ($r^2 = 0.28$, $P < 0.05$). Furthermore, all TDLNs collected from IDO$^+$ primary tumors were positive for IDO staining when we used immunohistochemical cut-off value of 10% for IDO$^+$ tumor cells. The mean IDO$^+$SIs in positive tumors and corresponding TDLNs were 26.47 ± 14.12% and 33.97 ± 13.91%, respectively. Contrarily, the mean IDO$^+$SIs in negative tumors and corresponding TDLNS were 5.56 ± 2.54% and 7.25 ± 3.43%, respectively. Therefore, comparing to the IDO$^-$ tumors, the TDLNs collected from IDO$^+$ tumors displayed higher level of IDO expression($P < 0.05$) which had no correlation with the pathological type and multiple receptors(ER/PR/Her2) status of the primary tumors. However, higher IDO$^+$SIs were observed in the metastatic TDLNs comparing to the nonmetastatic TDLNs, which were 34.41 ± 15.18% versus 21.45 ± 9.76% ($P < 0.05$). This result was consistent with the increase of IDO$^+$ myeloid cell-like karyocytes and cancer cells in metastatic TDLNs.

3.2. The Expression of IDO in Breast Cancer PTs Was Positively Associated with the Clinical Staging and Lymph Node Metastasis of Tumors. In order to evaluate the clinical significance of IDO expression in breast cancer PTs, a univariate analysis was performed between the IDO$^+$SI in PTs and corresponding clinical and pathological information of the same patient. As shown in Table 2, higher IDO$^+$SI correlated with more advanced clinical staging and more extensive TDLNs metastasis. The IDO$^+$SI in stage III breast cancer was significantly higher than those in stage II or stage I breast cancer, which

Table 2: The relationship between IDO expression and clinical pathological indexes.

	N	IDO$^+$SI (%)	P value
Age (years)			
<60	18	12.30 ± 8.35	0.465
≥60	8	15.11 ± 10.75	
Menstrual status			
Postmenopausal	14	15.02 ± 10.93	0.257
Nonpostmenopausal	12	10.83 ± 5.42	
Tumor diameter (cm)			
≤2	7	11.89 ± 6.55	0.211
2~5	15	13.43 ± 6.88	
>5	4	14.38 ± 7.69	
Clinical stage			
I	2	8.95 ± 3.79	0.034
II	20	11.72 ± 6.48	
III	4	22.47 ± 10.79*	
Pathological type			
Invasive Ductal Ca.	21	13.62 ± 9.39	0.223
Others	5	11.23 ± 6.25	
histological grade			
I	8	10.73 ± 6.45	0.324
II	10	12.29 ± 7.28	
III	8	13.71 ± 5.96	
TDLNs metastasis			
pN0	6	10.29 ± 5.23	0.046
pN1	14	11.42 ± 8.49	
pN2	4	15.98 ± 7.14	
pN3	2	28.35 ± 14.78*	
ER status			
(−)	12	15.76 ± 10.58	0.394
(+)	14	12.70 ± 8.83	
PR status			
(−)	13	13.18 ± 8.02	0.624
(+)	13	15.29 ± 9.31	
Her-2 status			
(−)	18	11.69 ± 8.46	0.457
(+)	8	14.34 ± 10.23	

*Statistically significant difference between the samples of advanced stage (stage III) and earlier stage (stage II and stage I), as well as the significant difference between the samples with more extensive LN metastasis (pN3) and less or no LN metastasis (pN0-2).

were 22.47 ± 10.79%, 11.72 ± 6.48%, and 8.95 ± 3.79%, respectively ($P < 0.05$). Similarly, the IDO$^+$SI in breast cancer with metastasis extended to N3 lymph nodes was significantly higher than those with metastasis limited to N2 and N1 lymph node or without lymph node metastasis, which were 28.35 ± 14.78%, 15.98 ± 7.14%, 11.42 ± 8.49%, and 10.29 ± 5.23%, respectively ($P < 0.05$). In contrast, there was no significant correlation between the IDO$^+$ SI in PTs and other clinical and pathological indexes, such as age, menstrual status, tumor diameter, pathological type, histological grade, and expression of ER, PR, or Her2.

All patients were followed up at a median of 5 years, and the overall survival (OS) and time to progression (TTP) were analyzed. The mean OS of the IDO$^+$ patients was shorter than that of the IDO$^-$ patients (59.50 ± 5.01 m versus 86.15 ± 3.22 m), but the difference was not statistically significant (P value = 0.145). Similarly, the mean TTP of the IDO$^+$ patients was shorter than that of the IDO$^-$ patients (46.84 ± 3.29 m versus 78.91 ± 2.79 m), but the difference was not statistically significant ($P = 0.147$). Although it is difficult to demonstrate an inverse correlation between clinical prognosis and the IDO status, possibly due to the small sample size, our results implied a comparably worse outcome in IDO$^+$ breast cancer patients.

3.3. The Expression of IDO in Breast Cancer PTs Was Positively Correlated with the Density of Tregs in PTs and TDLNs. The Foxp3$^+$ Tregs in PTs, TDLNs, benign disease, and normal adjacent tissues were detected using IHC staining method. Foxp3 protein was detected in the nuclei of lymphocytes infiltrated into PTs and TDLNs, but seldom in benign breast diseases and in normal breast tissues (Figures 2(a)–2(c)). The Foxp3$^+$ SIs in breast cancer PTs were significantly higher than those of benign breast diseases and normal breast tissues, which were 3.50 ± 1.04%, 0.71 ± 0.42%, and 0.55 ± 0.34%, respectively, ($P < 0.05$). In contrast, the Foxp3$^+$ SIs in the PTs were significantly lower than those in the TDLNs which was 6.13 ± 2.31% ($P < 0.05$). More Foxp3$^+$ Tregs infiltrated in the PTs with higher expression of IDO and corresponding TDLNs (Figures 2(d)–2(f)). Contrarily, in the breast cancer PTs with lower expression of IDO or absence of IDO expression, lower numbers of Foxp3$^+$ Tregs were detected (Figures 2(g)–2(i)). The scatter plots were generated to display the correlation between IDO expression and density of Foxp3$^+$ Tregs either in PTs or TDLNs. The results indicated that IDO expression in breast cancer was linearly correlated to the density of Treg in the PTs and TDLNs. Statistical analyses demonstrated that the IDO$^+$ SIs displayed a positive correlation with the Foxp3$^+$ SIs in PTs and TDLNs, with linear regression equations of $Y = 0.832 + 0.140X$ (Y: Foxp3$^+$ SIs in PTs; X: IDO$^+$SIs) ($r^2 = 0.449$, $P < 0.05$, Figure 2(j)) and $Y = 3.771 + 0.160X$ (Y: Foxp3$^+$ SIs in TDLNs; X: IDO$^+$SIs) ($r^2 = 0.324$, $P < 0.05$, Figure 2(k)), respectively.

3.4. The Proportion and Absolute Number of CD4$^+$CD25$^+$CD127$^-$ Tregs in CD3$^+$ T Cells Increased After Coculturing with IDO/CHO Cells. The mRNA and protein expression of IDO, as well as the catalytic activity of tryptophan have been determined in CHO/IDO cells as reported in our previous study [23]. The CD3$^+$ T cells isolated from PBMCs of breast cancer patients were cocultured with CHO/IDO and CHO/EGFP cells in complete medium supplemented with 10% FBS and 50 U/ml IL-2 for 7 days. The proportions and absolute number of CD4$^+$CD25$^+$CD127$^-$ Tregs in treated and untreated T cells were detected using flow cytometry. The proportion of Tregs in CD4$^+$ T cells increased from 3.43 ± 1.07% to 8.98 ± 1.58% after coculture with CHO/IDO cells, which is higher than that after coculture with CHO/EGFP cells (3.73 ± 1.12%) ($P < 0.05$,

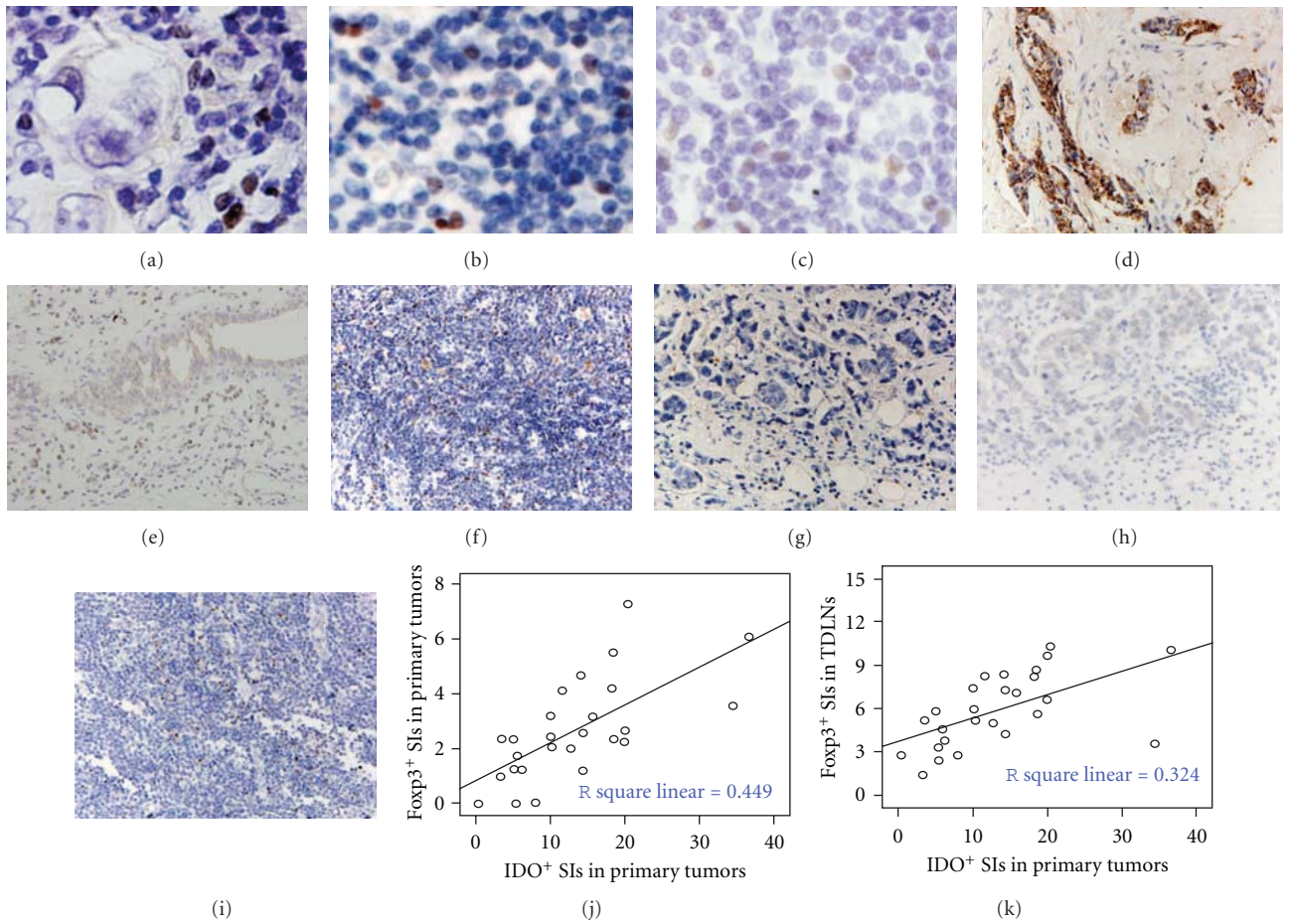

Figure 2: IDO expression in PTs was positively correlated with the density of Tregs in PTs and TDLNs. The Foxp3$^+$ Tregs in PTs, TDLNs, benign disease, and normal adjacent tissues were detected using IHC staining method. (a)–(c) The Foxp3 protein appeared in the nuclei of lymphocytes infiltrated into PTs (a) and TDLNs, including nonmetastatic TDLNs (b) and metastatic TDLNs (c). (d)–(i) In the PTs with higher expression of IDO (d), more Foxp3$^+$ Tregs infiltrated into the PTs (e) and corresponding TDLNs (f). In contrast, in the PTs with lower or no expression of IDO (g), less Foxp3$^+$ Tregs were detected in the PTs (h) and corresponding TDLNs (i). (j) and (k) Scatter plots were generated to display the correlation between IDO expression in breast cancer (IDO$^+$SIs in primary tumors) and the density of Foxp3$^+$ Tregs in PTs (Foxp3$^+$SIs in primary tumors) or in TDLNs (Foxp3$^+$SIs in TDLNs). The IDO$^+$SIs displayed a positive correlation with the Foxp3$^+$ SIs in PTs with a linear regression equation of $Y = 0.832 + 0.140\,X$ (j). Accordingly, the IDO$^+$SIs showed a similarly positive correlation with the Foxp3$^+$ SIs in TDLNs with a linear regression equation of $Y = 3.771 + 0.160\,X$ (k).

Figures 3(a)–3(c)). The absolute number of Tregs in CD3$^+$ T cells stimulated by CHO/IDO was 629 ± 110.6 cells/μL, higher than that in the coculture with CHO/EGFP cells (268 ± 80.6 cells/μL) and that in unstimulated control CD3$^+$ T cells (308 ± 96.3 cells/μL) ($P < 0.05$).

3.5. The Expression of Foxp3 in CD3$^+$ T Cells Was Upregulated at Both mRNA and Protein Levels After Coculturing with IDO/CHO Cells. The expression of Foxp3 gene at mRNA and protein levels in treated and untreated T cells were detected using qRT-PCR assay and Western Blot analysis. After 7 days of coculture, the relative mRNA amount of Foxp3 gene in the CD3$^+$ T cells stimulated by the CHO/IDO cells was 0.00056 ± 0.00012, which was significantly higher than that in the CD3$^+$ T cells stimulated by the CHO/EGFP cells (0.00023 ± 0.00005) and that in the unstimulated CD3$^+$ T cells control (0.00028 ± 0.00013) ($P < 0.05$, Figures 4(a) and 4(b)). Furthermore, Foxp3 expression was exclusively detected in the lysates of CD3$^+$ T cells stimulated by CHO/IDO cells, indicated by a 48 kD protein band reactive to a Foxp3-specific monoclonal antibody (Figure 4(c)).

4. Discussion

High level of IDO expression has been found in many malignant tumors, including colorectal cancer [24], endometrial cancer [25], lung cancer [26], ovarian cancer [27], and renal carcinoma [28]. However, its expression pattern in primary human breast cancer tissue has been seldomly reported. In this study we found that IDO expression at both mRNA and protein levels were significantly higher in breast cancer PTs and TDLNs than those in benign diseases.

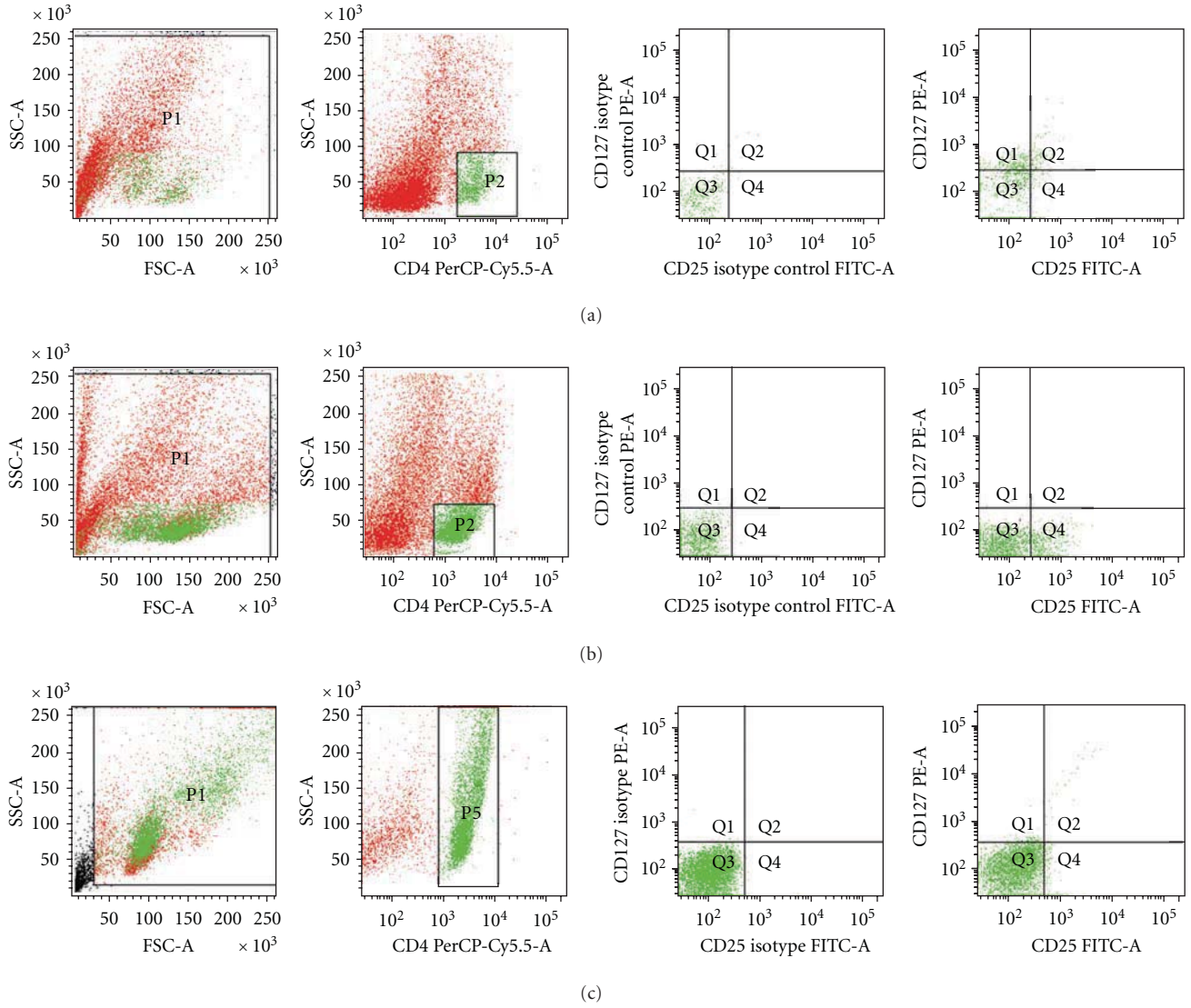

Figure 3: The proportion and absolute number of $CD4^+CD25^+CD127^-$ Tregs in $CD3^+$ T cells increased after coculture with IDO/CHO cells. $CD3^+$ T cells isolated from PBMCs of breast cancer patients were cocultured with CHO/IDO or CHO/EGFP cells for 7 days to allow induction of Tregs. The proportions and absolute number of $CD4^+CD25^+CD127^-$ Tregs were detected by flow cytometry. (a) The proportion of $CD4^+CD25^+CD127^-$ Tregs in the T cells treated with IDO^- CHO/EGFP cells (P2 region represents $CD4^+$ T cells; Q4 region represents $CD4^+CD25^+CD127^-$ Tregs). (b) The proportion of $CD4^+CD25^+CD127^-$ Tregs in the T cells treated with IDO^+ CHO/IDO cells (P2 region represents $CD4^+$ T cells; Q4 region represents $CD4^+CD25^+CD127^-$ Tregs). (c) The $CD3^+$ control T cells (P5 region represents $CD4^+$ T cells; Q4 region represents $CD4^+CD25^+CD127^-$ Tregs). The flow cytometry dot plots indicate data of one representative experiment. Each experiment was repeated at least 3 times.

IDO was mainly expressed in cancer cells in breast cancer PTs and expressed at lower levels in certain myeloid cell-like karyocytes in TDLNs and hyperplastic ductal cells in benign diseases, but not expressed in normal adjacent tissues. Our results demonstrated that the IDO^+SIs in breast cancer PTs were significantly higher in tumors of more advanced stages and with more extensive lymph node metastasis, which correlated with a comparably worse clinical outcome. These results suggest that IDO may play a pivotal role in promoting metastasis of breast cancer, as the IDO-positive breast cancer cells seem to have a higher potential in migrating to axillary lymph nodes than the IDO-negative ones.

The above results coincided with the previous reports that more extensive IDO expression in primary cancer tissues was associated with higher distant metastasis rate in clinic [24, 25]. A study by Sakurai et al. indicated that high expression of IDO in breast cancer correlated with clinical stage and may therefore play a critical role in immunosuppression in those patients [29]. However, the mechanisms involved in this pathogenesis process remain unknown. It is proposed that local T-cell-based immunotolerance induced by high level of IDO in the tumor microenvironment might be the predominant immunoregulatory mechanism facilitating tumor metastasis [24]. As a tryptophan catabolic enzyme,

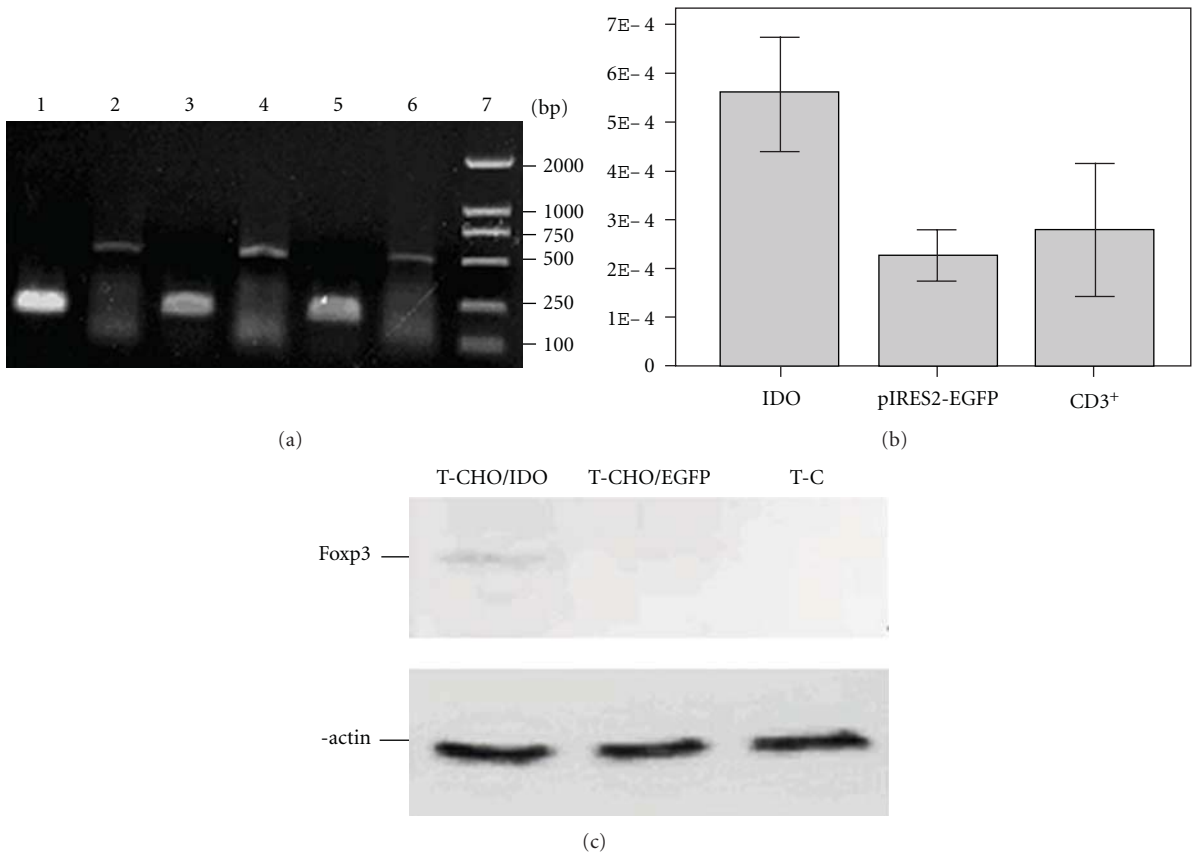

Figure 4: Foxp3 expression in $CD3^+$ T cells was upregulated both at mRNA and protein levels after coculture with IDO/CHO cells. The expression of Foxp3 gene at mRNA and protein levels in treated and untreated T cells were detected using qRT-PCR assay and Western Blot method. (a) After 7 day coculture, the relative mRNA level of Foxp3 in the $CD3^+$ T cells treated with the CHO/IDO cells was significantly higher than that in the $CD3^+$ T cells treated with the CHO/EGFP control cells or untreated $CD3^+$ T cells (lane 1: β-actin in the $CD3^+$ T control cells; lane 2: Foxp3 in the $CD3^+$ T control cells; lane 3: β-actin in the T cells treated with CHO/IDO cells; lane 4: Foxp3 in the T cells treated with CHO/IDO cells; lane 5: β-actin in the T cells treated with CHO/EGFP cells; lane 6: Foxp3 in the T cells treated with CHO/EGFP cells; lane 7: DL2000 Marker. (b) The mRNA amount of Foxp3 in the $CD3^+$ T cells stimulated by the CHO/IDO cells was significantly higher than that in the $CD3^+$ T cells stimulated by the CHO/EGFP cells and the unstimulated $CD3^+$ T cells control. (c) Foxp3 expression at protein level in treated and untreated T cells detected by Western Blot analysis. Foxp3 expression was exclusively detected in the cell lysates of $CD3^+$ T cells treated with CHO/IDO cells, indicating a 48 kD protein band reactive to a Foxp3-specific monoclonal antibody (lane 1: Foxp3 in $CD3^+$ T cells treated with CHO/IDO cells; lane 2: Foxp3 in $CD3^+$ T cells treated with CHO/EGFP cells; lane 3: Foxp3 in control $CD3^+$ T cells).

IDO and metabolites have been reported as key regulators in suppressing immune surveillance and inducing immunotolerance in several diseases [30]. Several mechanisms by which IDO contributes to immune escape have been identified. IDO suppresses proliferation of T cells by hampering cell cycle in mid-G1 phase [31]. IDO also promotes apoptosis of activated T cells which were more sensitive to Fas-dependent apoptosis after tryptophan deprivation [32]. Furthermore, IDO has been reported to inhibit T-cell-mediated immune response by directly inducing the differentiation of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ Tregs, or directly activating mature Tregs [33].

$CD4^+CD25^+$ Tregs are a subset of regulatory T cells with potent inhibitory effects on innate and adaptive immunity both in physiological and pathological status which play important roles in tumor evasion and metastasis [34, 35]. It is currently accepted that Foxp3 is the most specific marker in Tregs which plays crucial roles in the generation and function of Treg [36]. In our study, the $Foxp3^+$ SIs in breast cancer PTs were significantly higher than those in benign diseases and normal adjacent tissues, but lower than those in the TDLNs which showed the same pattern as the IDO^+SIs. Therefore, we studied the correlation between the expression levels of IDO and the density of Tregs both in breast cancer PTs and TDLNs. Our data demonstrated that in the IDO-positive breast cancer samples, more Tregs infiltrated into the PTs and TDLNs, compared to the IDO-negative ones. In addition, the expression of IDO in breast cancer PTs was positively linearly correlated to the density of Treg in the PTs and TDLNs in linear regression analysis. To find out if high level of IDO could induce amplification of $Foxp3^+$Tregs, we cocultured $CD3^+$ T cells with IDO^+ CHO(IDO/CHO) cells *in vitro*. We found that the proportion and absolute number of $CD4^+CD25^+CD127^-$ Tregs increased after coculturing $CD3^+$ T cells with IDO/CHO for 7 days in which Foxp3 expression was upregulated at both mRNA and protein levels.

These results implied that upregulated IDO in CHO cells might favor amplification of $CD4^+CD25^+CD127$ Tregs and induce increasing expression of Foxp3 both *in vivo* and *vitro* which coincided with the previous report that the long-term effect of the catabolic products of tryptophan is to enable the regulatory function of $CD4^+CD25^-$ T cells by inducing Foxp3 expression and secreting inhibitory cytokine TGF-β [37].

It is has been indicated that the interaction between IDO and Tregs is a mutual effect, in which high level of IDO promotes the differentiation, activation, and maturation of Tregs; conversely, the CTLA4 constitutively expressed on $CD4^+CD25^+$ Tregs significantly stimulates synthesis and increases enzyme activity of IDO by binding to CD80/CD86 on dendritic cells (DCs) [38]. This theory was supported by the observations from Munn's group which indicate that overexpression of IDO in antigen-presenting cells (APCs) was the major cause of tumor-derived immune tolerance in local lymph nodes of patients with breast cancer or melanoma [39]. Consistently, in this study we also observed that some IDO high-expressing myeloid cell-like karyocytes in TDLNs displayed positively linear correlation to the IDO expression on cancer cells in PTs, which might participate in the mutual cross-talk between IDO^+ cancer cells and $Foxp3^+$ Tregs and further magnified the immunosuppressive cascade triggered by IDO.

However, Jacquemier et al. reported an opposite effect of IDO in medullary breast cancer (MBC), a subtype of basal-like breast cancer different from invasive carcinoma, in which high expression of IDO in stromal or epithelial cells was associated with large amount of lympoid infiltrate and a favorable clinical outcome of patients [40]. This report, however, stated that the beneficial prognosis of IDO^+ tumors was exclusively observed in basal-like breast cancer, but not in other subtypes of breast cancer. Therefore, the discrepancy between this previous study and ours may be attributed to the different pathological subtypes of breast cancer examined. In our study, most samples were invasive ductal or lobular carcinomas, and no basal-like breast cancers are included. Our conclusion is consistent with the study of Mansfield et al. using 47 cases of breast cancer samples, including 25 invasive ductal carcinoma and 18 invasive lobular carcinoma, where IDO^+ sentinel lymph nodes accompanied by infiltration of $Foxp3^+$ Tregs imply lymph node metastasis of breast cancer, and are therefore regarded as a negative prognostic factor [12].

In conclusion, our study implied that upregulation of IDO in breast cancer cells might inhibit local immune surveillance by favoring amplification and infiltration of $CD4^+CD25^+$ Tregs in the tumor microenvironment and thus promote metastasis and relate to a bad prognosis. Therefore, novel and efficient IDO-targeted therapies may provide a new strategy of breast cancer treatment.

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Review Article

PI3K Functions in Cancer Progression, Anticancer Immunity and Immune Evasion by Tumors

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The immunological surveillance of tumors relies on a specific recognition of cancer cells and their associate antigens by leucocytes of innate and adaptive immune responses. However, a dysregulated cytokine release can lead to, or be associated with, a failure in cell-cell recognition, thus, allowing cancer cells to evade the killing system. The phosphatidylinositol 3-kinase (PI3K) pathway regulates multiple cellular processes which underlie immune responses against pathogens or malignant cells. Conversely, there is accumulating evidence that the PI3K pathway is involved in the development of several malignant traits of cancer cells as well as their escape from immunity. Herein, we review the counteracting roles of PI3K not only in antitumor immune response but also in the mechanisms that cancer cells use to avoid leukocyte attack. In addition, we discuss, from antitumor immunological point of view, the potential benefits and disadvantages arising from use of anticancer pharmacological agents targeting the PI3K pathway.

1. PI3K Pathway in Tumor Development and Progression

The PI3K signaling pathway regulates the activities of a broad range of downstream molecular effectors, which in turn act synergistically to mediate a number of cell behaviors and properties in both normal and pathological conditions. An overview of the involvement of PI3K in these conditions is summarized in Figure 1. Three classes of PI3K enzymes have been defined. The class I is the most intensely studied and includes p110α, β, γ, and δ catalytic isoforms, which are controlled by coupling with their proper regulatory isoforms (p85 and p101) to effect their lipid kinase activity [1]. The PI3K activation in terms of signaling response varies according to the type of stimulus. For example, p110α and δ are recruited and activated at the plasma membrane upon activation of tyrosine kinase receptors (TKRs) whereas p110γ requires engagement of G-protein-coupled receptors (GPCR). Conversely, p110β can be activated by both TKRs and GPCR [2]. Nevertheless, recent data reveal a more complex regulation for p110δ, as this isoform is linked to specific GPCRs signaling [3]. Once activated, PI3K enzymes catalyze the phosphorylation in position 3 of the inositol ring of phosphoinositides, resulting in the generation of 3-phosphoinositides, mainly the phosphatidylinositol-3-trisphosphate (PIP3). These lipids act as docking sites for the recruitment at plasma membrane of protein-bearing pleckstrin homology (PH) domain such as Akt/PKB, PDK1, BTK, and PLCγ. Once bound to PIP3 lipids, these proteins turn activated and signal to a wide array of downstream effectors that ultimately leads to multiple cellular responses [4–8]. This signaling cascade can be antagonized by the action of the phosphatase and tensin homolog (PTEN), a widely recognized tumor suppressor which dephosphorylates the PIP3 [9].

The oncogenic transformation of cultured cells as well as the progression of a variety of tumors *in vivo* has been reported to be induced by mutations or overexpression of p110 isoforms. For example, cultured cells undergo transformation when a catalytically hyperactive mutated isoform of p110α is ectopically expressed whereas p110β, γ, and δ are oncogenic only when overexpressed [10]. Mutations

of p110α disrupting the interaction with the p85 subunit can also induce oncogenic transformation in the absence of the receptor activation [11, 12]. The oncogenic role of p110α has been previously demonstrated in ovarian cancers, where an increased number of PIK3CA gene copies was observed. This was correlated with the overexpression of the p110α subunit that results in an augmented activity of PI3 kinase [13]. Mutations of the PIK3CA gene were found with high frequency in colon, brain, breast, liver, and gastric cancers suggesting an involvement of isoform p110α in cancer [14, 15]. The activity of p110β, but not p110α, was shown to be essential in promoting PTEN-driven tumorigenesis in an animal model of prostate tumor. Importantly, Akt is shown to be a mediator of p110β-dependent tumorigenesis [16]. This finding was supported by a complementary approach based on the transgenic expression of a constitutively activated p110β in prostate of mice. In this study, overexpression of this hyperactive isoform drives the formation of a intraepithelial neoplasia [17]. p110γ has been recently shown to positively regulate tumor cell proliferation in HCC and pancreas cancer [18, 19]. In addition, pharmacological inhibition of p110γ in medulloblastoma cell lines led to an impairment in cell proliferation and sensitized them to cisplatin treatment [20]. A role for p110δ in sustaining neuroblastoma growth has been recently reported. Both primary neuroblastoma cells and tissues displayed an overexpression of p110δ and p85α in comparison with the normal adrenal gland tissue. Moreover, knockdown of both p110α and δ isoform triggered defective cell growth, whereas only p110δ knockdown affected cell survival, via lowering the expression of the Bcl-2 antiapoptotic family proteins [21]. The progression of multiple B-cell malignancies was found to be dependent on a constitutive activation of p110δ [22]. In particular, increased levels of p110δ were found in blast cells from patients with acute myeloid leukemia (AML). In addition, pharmacological targeting of p110δ resulted in an inhibition of the AML cell proliferation [23]. Finally, the PI3K signaling pathway was shown to be constitutively activated in chronic lymphocytic leukemia B cells (CLL). Moreover, dysregulation of the PI3K signaling pathway prevents CLL cell survival by inducing apoptosis through caspase-3 activation [24].

2. Role of PI3K Pathway in Immune Response to Tumors

Different cell types are involved in immune response to tumors. Natural killer (NK) cells intervene in a first-line defense against tumor cells. These lymphocytes constantly comb the cell microenvironment, where they check the expression level of MHC class I at the membrane of their targets, which can be reduced as a result of viral infection or oncogenic transformation. NK cells are cytotoxic against cells that fail to expose MHC class I on their surface, thanks to NK-inhibiting receptors for MHC class I that exist on cell membrane of NK cells [25]. Once activated, these receptors (belonging to three families named KIRs, ILTs, and NKG2A/CD94) inhibit for the cytolytic activity of NK cells by

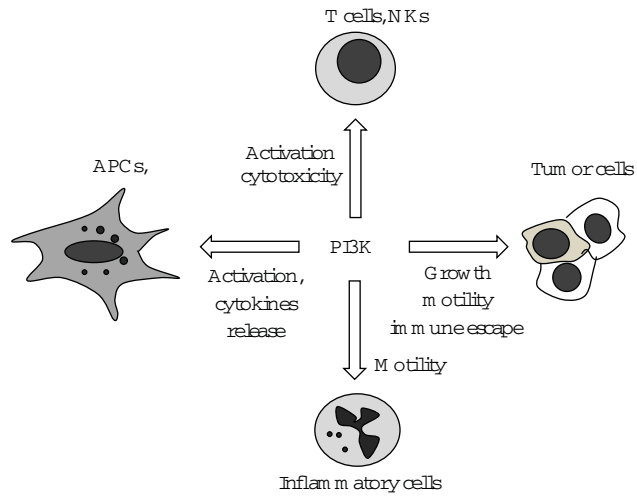

Figure 1: Schematic model of the PI3K signaling pathway involved in the regulation of a broad range of cellular activities in both immune system and cancer.

binding to HLA class I. Beside inhibitory receptors, NK cells bear different activating receptors which elicit their cytolytic effect on target cells after binding to a broad range of ligands. One of the best studied among the activating receptors of NK cells is the C-type lectin-like superfamily member NKG2D, which also occurs in CD8 T cell in humans. This receptor is a transmembrane glycoprotein which binds some known ligands (MHC class I chain-related molecules (MIC) MIC-A, -B, and ULBP) which are little expressed on the surface of normal cells but can be increased in transformed or virus-infected cells [26]. The antigen-presenting cells (APCs), primarily dendritic cells (DCs) and macrophages, can prime specific CD4+ and CD8+ T-lymphocyte-mediated responses to cancer cells, thanks to their ability to recognize tumor-associated or specific antigens, and present antigen-derived peptides in the MHC class II. The generation of tumor addressed T-cell clones is driven by stimulatory signals occurring when immunological synapses form between APCs and T-cells. DCs and macrophages secrete cytokines, such as IL-12, IL-15, IL-18, necessary for induction of NK and T-cell immunity. IL-12 leads to differentiation of CD4+ cells in Th1 subtype which is effective in tumor rejection. Th1 cells help expand the population of CD8+ cytotoxic T lymphocytes that can directly destroy tumor cells [27]. NK cells release IFNγ in response to stimulation by both mature DCs secreted IL-12 and cell-to-cell contact with DCs [28]. Also, IL-12 stimulate Th1 and CD8+ to secrete IFNγ [29] which in turn promotes a wide array of host responses to tumors [30], including the activation of CD8+ cells [31] and the recruitment of NK cells within the tumor [32].

Chronic inflammation is thought to underlie the onset of several cancers. Several reports demonstrate that PI3Ks activity is essential in regulating chemokine production by leukocytes as well as directional migration of these cells during the inflammatory response. For example, studies carried out *in vivo* using models for inflammation show that p110γ

is required to allow chemotactic migration of neutrophils, macrophages, and effector CD8 T cells to inflammatory sites [33, 34]. During lung inflammation, recruitment of eosinophils to the bronchial epithelium, together with the repulsion of neutrophils exerted by chemokine gradients rely on the activity status of PI3K signaling in these leukocytes [35]. Moreover, the release of IL-8, Mip-1α, and Mip-1β by neutrophils in response to LPS and TNFα require the activity of p85/p110δ complex [36].

Studies performed in mice using loss of function of p110 isoforms and their related regulatory subunits demonstrate a crucial role for PI3K in development of immune cells involved in tumor clearance. The PI3K/Akt-dependent mTOR pathway is reported to be essential in GM-CSF-induced differentiation of DCs from monocytes [37]. Webb et al. demonstrate that the functions of p110γ and p110δ PI3K isoforms are required for T-cell development [38]. In a study recently published, Kerr and Colucci report the need for p110δ to achieve NK cell maturity, as well as a cooperation between p110γ and p110δ isoforms in establishing the repertoire of inhibitory receptors of the Ly49 family in mice (the homolog family in humans is KIR) [39]. Other authors have previously shown that the achievement of NK cell subsets maturity is impaired in mice either expressing lipid kinase-inactive p110δ or lacking regulatory p85α/p55α/p50α subunits. Moreover, inactive p110δ or p85α/p55α/p50α depletion was shown to result in significantly compromised NKG2D, Ly49D, and NK1.1 receptor-mediated cytokine and chemokine generation in NK cells, even if the NK-mediated cytotoxicity against tumor cells was affected only in mice lacking p85 regulatory subunit [40, 41].

An involvement of the PI3K/Akt pathway has been reported in the immune recognition of tumor cells. For example, in NK cells, the NKG2D-associated adapter protein DAP10 undergoes Tyr phosphorylation in its cytoplasmic tail following interaction between NKG2D and activating ligands. This allows DAP10 to anchor to either the p85 subunit of PI3K or to the adaptor Grb2, leading to PKB/AKT or MAP kinase signaling activation, respectively. These signaling cascades enable cytolytic activity and chemokine production by NK cells [42–44]. Furthermore, the small Ras family GTPase Rap1 is activated downstream of NKG2D engagement in a PI3K- and CrkL-dependent manner and is required for NK cell/target cell conjugate formation, NK cell polarization, and NKG2D-dependent cellular cytotoxicity [45]. Different activating receptors, other than NKG2D, can lead to NK cytotoxicity against tumor cells using the adapter DAP12, instead of DAP10, for PI3K pathway stimulation. DAP12 is tyrosine phosphorylated upon tumor cell ligation allowing binding of DAP12 to Syk kinase, which in turn activates the signaling pathway PI3K, Rac1, PAK1, and ERK leading to the lytic cascade of NK cells [46].

The engagement of NKG2D through coculturing human NK cells with MICA-bearing tumor cells leads to a PI3K-dependent increase of IFNγ secretion by NK cells. This is an additional effect to IFNγ release upon treatment of the same cells with IFN-α, IL-12, and specific agonists for TLR3- and TLR7-activating receptors [47]. These findings support the relevant role of the PI3K pathway as a mediator of the adaptive immune response against tumors by activated NK cells. The role of PI3K in the APCs production of IL-12 remains controversial. A report by Ohtani and coworkers show a complex cooperation between the PI3K-downstream GSK3 and mTOR pathways in the regulation of IL-12 secretion as a consequence of TLR activation by LPS on DCs. These authors show that GSK-3 and mTOR activities promote and reduce IL-12 production, respectively. However, the overall effect of LPS on DCs is to reduce IL-12 secretion, since PI3K activation blocks GSK-3 function while enhancing the mTOR signaling [48]. Conversely, other studies show an overall increased IL-12 production by human macrophages and DCs, upon LPS stimulation which depends on the activation of p110β isoform of PI3K [49].

The CD28-dependent costimulating signals required for the full activation of T cells by APCs are mediated partially by PI3K functions. CD28 undergoes tyrosine phosphorylation in its cytoplasmic tail upon binding to APCs costimulatory ligand B7. This binding recruits p85 subunit at the cell membrane through the interaction between SH2 domains of p85 and the phospho-tyr docking sites of CD28. As a consequence, p85 binds to the catalytic subunit p110 that activates PKCθ, which is capable of preventing stress-induced apoptosis of T cells [50].

3. The PI3K/Akt Pathway Is Involved in Escape of Tumors from Immunological Surveillance, Immune Suppression, and Acquired Leukocyte-Like Properties by Cancer Cells

The PI3K pathway can be responsible, to a certain extent, for transformed cells escaping immunity. Examples of some of the immune escape mechanisms by cancer involving the PI3K signaling pathway is summarized in Figure 2. A reduced NKG2D expression and function in NK cells following chronic exposure to NKG2D ligands and/or soluble forms of MIC (sMIC) leads to a immune surveillance failure [51]. This occurs in chronic myeloid leukemia, where the BCR/ABL fusion oncoprotein is shown to positively regulate the expression of MICA/B at the translational level via a PI3K-dependent mechanism in the BCR/ABL+ cell line K562 [52]. Cancer cells can also escape immune surveillance by developing a *de novo* expression on their surface of some molecules which are normally present in immune cells, thus allowing them to be recognized as normal. Melanoma cells often express MHC II, and this histological condition is associated with poor prognosis. Melanoma-infiltrating T cells express the lymphocyte activation gene 3 (*LAG-3*), which is a natural ligand for MHC II. Activation of MHC II on melanoma cells promotes resistance against FAS-mediated or drug-induced apoptosis via a mechanism based on MAPK/Erk and PI3K/Akt pathways [53]. Noh and coworkers supported furthermore the role of PI3K/Akt axis in the setting of immune escape. An immune-resistant human papillomavirus type 16 (HPV-16) E7-expressing tumor cell line was generated by these authors. A hyperactivation of Akt, after E7-specific vaccine administration, was found to

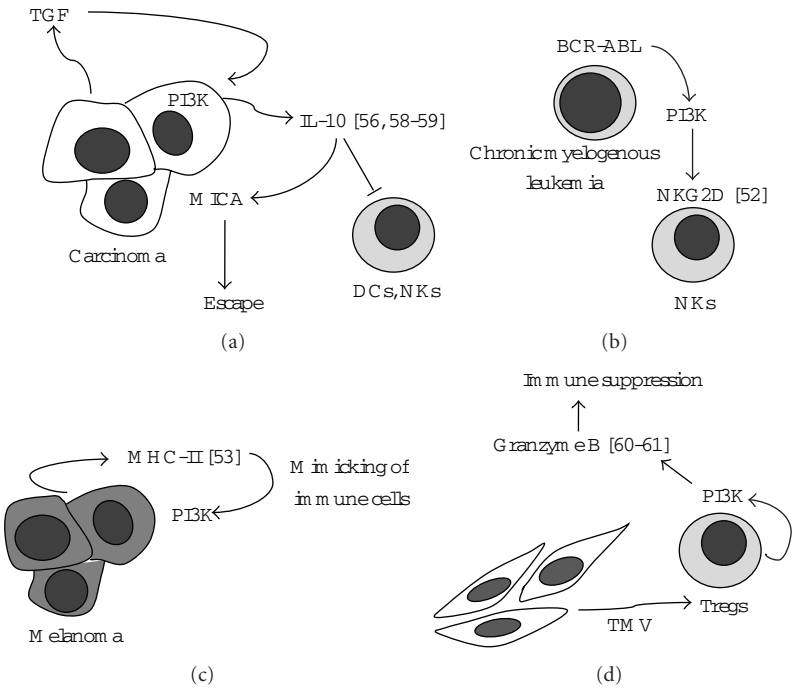

Figure 2: Examples of the major immune escape mechanisms of different types of cancers displaying the involvement of the PI3K signaling pathway. : upregulation; : downregulation; : activation/secretion; : inhibition.

be responsible for the increased resistance of these cells to CD8(+) T-cell-mediated apoptosis [54].

In addition, cancer can overcome immunity through a metabolic enhancement arising from *de novo* expression of pathways that leukocytes use in anticancer processes. Unexpectedly, a *de novo* expression of the NKG2D/DAP10 complex has been reported in human cancer cells both *in vitro* and *in vivo*. Notably, in this study, the authors demonstrate a complementary function between NKG2D/DAP10 and its MICA ligand, resulting in a self-sufficiency of cancer cells in activating of PI3K/Akt-dependent NKG2D downstream signaling. Therefore, the activation of Akt-downstream mTOR/S6K/4EBP1 signaling axis upon NKG2D/DAP10 stimulation is shown to promote a sustained cancer progression via an increased energetic metabolism [55].

Cancer cells can drive immune suppression by multiple mechanisms, including the secretion of immune-suppressive cytokines and chemokines, such as TGFβ and IL-10 [56], or FasL expressing microvesicles (TMV) which induce lymphocyte apoptosis [57]. The PI3K signaling is reported to mediate cellular responses upon exposure to these microenvironmental factors. The pleiotropic cytokine TGFβ1 increases the expression of IL-10 and MCP-1 in melanoma cells, through a crosstalk between Smad, PI3K/AKT, and BRAF-MAPK signaling pathways. IL-10 induces decreased MICA expression on melanoma cells in an autocrine loop and blocks the antitumor functions of DCs and NK cells. MCP-1 recruits monocytes, which in turn secrete TGFβ1, FGF, and proangiogenic factors (VEGF), and then differentiate into macrophages. The cooperation of these processes can boost the progression of melanoma [58, 59]. Cancer cells can also employ a more indirect mechanism to inhibit immune surveillance by enhancing the immune-suppressive function of T-regulatory (Treg) cells. TMV secreted by cancer cells can convert CD4(+)CD25(−) T cells into CD4(+)CD25(+)FOXP3(+) Treg, while increasing the expression by these cells of immune-suppressive factors, such as FasL, IL-10, TGF-β1, CTLA-4, granzyme B, and perforin [60]. *In vitro* studies demonstrate that the PI3K-mTOR pathway is required for the Granzyme B release by Treg, upon prolonged stimulation of TCR and CD28, synergically with IL-2 stimulation [61]. Moreover, Tregs derived from p110δ defective mice show an impaired suppression function *in vitro* and fail to secrete IL-10 [62].

A central role of PI3K in processes involving leukocytes motility (inflammation, adaptive immune responses, tumor infiltration) has been widely documented [63]. For example, PI3K isoform p110γ and p110δ are both required to mediate chemotaxis of NK cells induced by CXCL12 and CCL3 during pregnancy. In addition, p110δ is involved in S1P and CXCL10-mediated chemotaxis and in NK cell tissue distribution and tumor infiltration [3]. Antigen-activated p110γ-deficient CD4+ lymphocytes exhibit impaired F-actin polarization and migration into peripheral inflammatory sites in response to stimulation *ex vivo* with the CCR4 ligand CCL22 [64]. Using a mechanism PI3K dependent, cancer cells can also increase their malignancy by "emulating" some immune cell chemotactic responses. For example, the chemokine CCL5 (also called RANTES), previously known as a motility factor for some leukocytes during inflammation, can induce migration and metastasis of human cancer cells thanks to developing a *de novo* expression of CCL5 receptor

Table 1: Main effects of the PI3K and VEGFR inhibitors on immune cells.

PI3K inhibitors	p110 isoform	VEGFR2/3 inhibitors	Effect	Ref.
PIK-75	α		Reduced production of TNF-α and IL-6, reduced expression of human endothelial cell adhesion molecules (E-selectin, ICAM-1, and VCAM-1), and human monocyte-endothelial cell adhesion.	[79]
AS-605240	γ		Reduced numbers of infiltrated proinflammatory macrophages and T cells.	[80, 81]
AS041164	γ		Reduced RANTES-induced chemotaxis/recruitment.	[82]
CAL-101	δ		Apoptosis of CLL cells and decreased production of various inflammatory and antiapoptotic cytokines by activated T cells.	[72]
IC87114	δ		Reduced antigen-induced airway infiltration of inflammatory cells, secretion of T(H)2 cytokines in lungs, and inhibition of monocytic integrin activation during diapedesis.	[83]
		SU5416	Reduced IFNγ secretion by CD4+ CD45RO+ T cells.	[84]
		E7080	Reduced lymphocytes in tumor.	[85]
		TSU68	Decreased expression of CXCL1 (by cancer cells) and IL-12 and reduced neutrophil migration into tumor.	[86]

(CCR5) at their surface, which is not present in non-cancerous cell lines. Tang et al. have demonstrated that chondrosarcoma cells express CCR5 and can sense CCL5 resulting in increased cell migration and metalloproteinases-3 secretion. The PI3K and NF-κB pathways have been shown to play an essential role in this scenario [65].

4. Pharmacological Inhibition of PI3K in Cancer Treatment and Antitumor Immune-Response

The choice of suitable anticancer pharmacological agents requires a careful assessment of their side effects on the immune defense against cancerous cells. Although the role of a dysregulated PI3K pathway in the development of malignancy is well documented, a cancer treatment featuring PI3K inhibition might be deleterious to the immune response to tumors. In advanced renal cell cancer (RCC), treatment with Sorafenib but not Sunitinib can impair antitumor immune responses, through inhibiting PI3K and ERK phosphorylation in NK cells, thus, impeding the release by these cells of cytokines activating adaptive immune responses (i.e., IFNγ), as well as killing tumor cell targets [66]. However, this is in contrast with of antitumor immune enhancement effect reported for Sorafenib in hepatocellular carcinoma (HCC). This drug has been reported to downregulate the expression of metalloproteinase ADAM9 in HCC cells, which is involved in proteolytic cleavage of MICA, thereby, allowing this ligand to be displayed on the HCC cell surface for NK recognition [67]. A study by Ghebeh and coworkers provides evidence of detrimental effects arising from a combination of inhibition of the PI3K/AKT pathway and chemotherapy in an *in vivo* xenograft mouse model of cancer treatment. Indeed, the anthracycline doxorubicin has been shown to mediate nuclear translocation of the T-cell inhibitory molecule, B7-H1 (PD-L1, CD274), and phosphorylated AKT in breast cancer cells in a PI3K-dependent manner, restoring immune surveillance. Interestingly, these authors show an additional role for B7-H1 in preventing apoptosis in breast cancer cells, thus, providing a link between immune resistance and chemoresistance [68]. In CML therapy, in addition to diminishing the expression of ligands for the activating immunoreceptor NKG2D by tumor cells, the BCR/ABL-inhibitor Dasatinib can impair NK cell reactivity as well as IFNγ production. Dasatinib treatment was shown to inhibit the phosphorylation of PI3K and ERK, which are crucial for NK cell cytolytic activity [69]. The option of using p110 isoform-specific inhibitors for cancer treatment must be considered with care, as the function of a single isoform can be dually involved in promoting both tumor progression and antitumor immunity. A failure in NK cell-mediated clearance of cancerous cells has been reported in studies using p110δ knock-out mice. Although this isoform promotes the progression of leukemia, p110δ depletion results in a defective ability of NK cells to degranulate and kill a large variety of target cells [70]. Nevertheless, the use of p110δ inhibitor CAL-101 has recently proven effective in an *ex vivo* model of CLL, a disease that shows a high PI3K activity [71]. CAL-101 induces apoptosis of malignant cells without affecting normal T cells or NK cells. However, the effect of CAL-101 on NK or CD8+ and cell-mediated cytolytic functions of these cells has not yet been explored [72]. This evidence supports the notion that therapeutic benefits arising from targeting PI3K isoforms could depend on a balance between the benefit of purging cancer cells and the disadvantages of immunological impairment.

Evaluation of whether the inhibition of PI3K enzymes might lead to benefits in cancer therapy should also be based on the stage of disease when starting treatment. The sustained activation of lymphocytes in chronic inflammation, which underlies the development of several cancers, relies on PI3K activity in some cases. For example, p110γ isoform has been shown to drive the onset of colitis-associated tumors, due to its role in the activation and infiltration of myeloid cells and recruitment of T cells to the colon [73]. An anti-inflammatory therapy based on p110γ inhibition to prevent the onset of colitis-associated tumors could interfere with

antitumor immunity when an early stage cancer is already developing, as the NK cells reactivity depends strongly on the activity of this isoform [3].

A quest for PI3K inhibitors with a selective action on malignant cells without affecting immune cells may reveal compounds that could offer a promising anticancer strategy while preserving anticancer immunological reactivity. For example, Honokiol, a plant-derived compound, was shown to be efficient in downregulating levels of phospho-S6 and B7-H1 in tumor cells via PI3K/mTOR pathway, thus, impairing the immune resistance of glioma, breast, and prostate cancer cell lines, while having no effect on critical proinflammatory T-cell functions. This does not occur with classic PI3K/mTOR inhibitors, including LY294002, wortmannin, AKT inhibitor III, and rapamycin [74]. Conversely, a selective therapy based on a specific pharmacologically induced T-cell PI3K/AKT pathway would prevent the tumor-induced death/suppression of immune cells potentially engaged in tumor clearance. Apoptosis induced *in vitro* on CD8(+) T-cells by tumor-derived microvesicles expressing FasL has been successfully inhibited by treating these lymphocytes with cytokine-based biologic agents, such as IRX-2, which, like IL-2, IL-7, or IL-15, block the apoptotic machinery through Akt activation [75].

5. Role of Immunomodulatory Drugs Currently Implemented for the Treatment of Tumor and Effect of PI3K Inhibitors on Immune Cells

A number of immunomodulatory drugs are currently under investigation for their anticancer activity. For instance, a novel strategy for treatment of advanced malignancies suggests the use of bispecific T-cell-engaging (BiTE) antibodies which cluster T-cells and cancer cells, and this results in an enhanced cytotoxic activity toward tumor cells. The recently developed therapeutic antibody Blinatumomab has a dual specificity for CD19 and CD3. Promising responses arose from the use of Blinatumomab in B-cell non-Hodgkin's lymphoma (NHL) and B-precursor acute lymphocytic leukemia (ALL) [76]. PF3512676 can activate TLR9 on plasmocytoid dendritic cells, thus, leading to increased expression of class I/II MHC costimulatory molecules and secretion of cytokines/chemokines that enhance antitumor NK cell activity. Lenalidomide can improve host immunity against tumor cells by stimulating LPS-induced IL-10 as well as costimulators of CD8+ T cells. Furthermore, it induces IL-2 and IFNγ delivery by T cells, resulting in activation of NK cells [77]. However, a hyperactive PI3K pathway in tumor cells can counteract the beneficial effects of immunomodulatory agents used for enhancing antitumor immune responses. p110δ isoform was shown to promote activation of CLL cells, as well as VEGF and FGF expression in response to lenalidomide [78]. With regard to VEGF and PI3 kinase downstream Signaling, it is worthy to mention that both VEGF and PI3 kinase inhibitors have an effect on the immune cells. Inhibitors and the main effects on the immune cells are summarized in Table 1.

Immunomodulators that enhance immune response against low immunogenic cancer-specific antigens during vaccine-mediated therapies are currently under development. One example is the use of multifunctional immunomodulator SA-4-1BBL during vaccination against the E7 HPV-associated oncoprotein for treatment of cervical cancer [87]. Another example is provided by IFNα that possess advantageous immunomodulatory properties including activation of DCs. However, the use of this chemokine in cancer immunotherapy is limited since it can cause autoimmune disorders [88]. Another strategy is to employ immune-directed (rather than antitumor) monoclonal antibodies (mAbs) targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4), an inhibitory molecule on T cells. Ipilimumab and tremelimumab, two anti-CTLA-4 mAbs, have shown a better clinical antitumor response than the traditional tumor-targeting mAbs [89].

Immunomodulatory oligonucleotides (IMOs) represent a new class of compounds with anticancer properties. Their efficacy in inhibiting tumor formation has been demonstrated alone or in combination with chemotherapeutic agents both *in vitro* and *in vivo* in breast, prostate, and nonsmall cell lung cancer. TLR9 was recently found to be expressed in cancer cells apart from that in APCs. The anticancer activity of TLR9 as a receptor for IMOs and mediator of IMOs has also been described [90–92]. Thalidomide and its analogs inhibit angiogenesis indirectly by blocking the action of TNF-α, while activating costimulation in T cell. These drugs are employed alone or combined with chemotherapeutics in the treatment of some malignancies, including lung cancer and multiple myeloma [93, 94].

6. Concluding Remarks

Tumor growth may be the result of tumor proliferation and tumor-induced failure of immunity in killing cancer cells [95]. The PI3K signaling pathway is required in multiple processes, including not only cancer progression, escape of cancer cells from immunological surveillance, immune suppression and acquisition of leukocyte-like properties by cancer cells but also anticancer immune responses. This assumption raises concerns about the proper use of PI3K-targeting inhibitors. On one hand, the pharmacological inhibition of PI3Ks in cancer would be beneficial because of the blockage of tumor growth and immune-suppressive function mediated by PI3K. On the other hand, it could be hazardous since the PI3K signaling pathway is crucial in antitumor immunity. Therefore, to minimize deleterious effects, a therapeutic inhibition of PI3Ks should be selective as much as possible on targeting of cancer cells without having inhibitory effect on the immune system.

Abbreviations

PI3K: Phosphatidylinositol 3-kinase
NK: Natural killer
HLA: Human leukocyte antigen
KIR: Killer-cell immunoglobulin-like receptor

ILT: Immunoglobulin-like transcript
APC: Antigen presenting cell
IL: Interleukin
LPS: Lipopolysaccharide
TNFα: Tumor necrosis factor α
GM-CSF: Granulocyte macrophage colony-stimulating factor
DC: Dendritic cell
TLR: Toll-like receptor
mTOR: Mammalian target of rapamycin
MICA/B: MHC class I-related chain A/B
ULBP: UL16-binding protein
MCP-1: Monocyte chemoattractant protein-1
TGFβ: Transforming growth factor β
CXCL: CXC chemokine ligand
CCL: CC chemokine ligand
CCR: CC chemokine receptor
ADAM9: Disintegrin and metalloproteinase domain-containing protein 9
IFNγ: Interferon γ
CML: Chronic myelogenous leukemia
CLL: Chronic lymphocytic leukemia
TMV: Tumor-derived microvesicles
VEGF: Vascular endothelial growth factor.

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Review Article

Recent Advance in Antigen-Specific Immunotherapy for Acute Myeloid Leukemia

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Relapse after chemotherapy is inevitable in the majority of patients with acute myeloid leukemia (AML). Thus, it is necessary to develop novel therapies that have different antileukemic mechanisms. Recent advances in immunology and identification of promising leukemia-associated antigens open the possibilities for eradicating minimal residual diseases by antigen-specific immunotherapy after chemotherapy. Several methods have been pursued as immunotherapies for AML: peptide vaccines, granulocyte-macrophage colony-stimulating factor-secreting tumor vaccines, dendritic cell vaccines, and adoptive T cell therapy. Whereas immunogenicity and clinical outcomes are improving in these trials, severe adverse events were observed in highly avid engineered T cell therapies, indicating the importance of the balance between effectiveness and side effects in advanced immunotherapy. Such progress in inducing antitumor immune responses, together with strategies to attenuate immunosuppressive factors, will establish immunotherapy as an important armament to combat AML.

1. Introduction

The immune system has an exquisite ability to specifically kill cells that express particular antigens. This specificity is the heart of immunotherapies that eliminate tumor cells without damaging normal cells. Recent advances in immunology research have revealed many facets in the immune system that are important to develop tumor immunotherapy. At the same time, recent studies have identified many promising acute-myeloid-leukemia- (AML-) associated antigens that can be targeted by immunotherapy. The combination of such advancement may enable antigen-specific immunotherapies to be established as a viable choice of therapy for AML.

Here we review recent advance in antigen-specific autologous immunotherapy for AML and raise several issues to overcome in order to improve clinical efficacy in the future. This review excludes graft-versus-leukemia (GVL) effects exploited in allogeneic hematopoietic stem cell transplantation, which are mainly allogeneic immune reactions.

2. Importance of Immunotherapy for AML

Cytotoxic chemotherapy and allogeneic transplantation are practically the only two modalities to treat AML. Chemotherapy has inevitable limitations of effectiveness due to chemoresistance in the majority of AML patients, except for a small fraction of patients with favorable karyotypes. Allogeneic transplantation is inherently accompanied by a variety of life-threatening complications related to graft-versus-host disease (GVHD), which limits candidates to younger and fit patients. Given such situations, immunotherapy may potentially play an important part in the treatment for AML, mainly from the following two standpoints.

2.1. Treatment of Minimal Residual Disease after Chemotherapy. Although it is possible to achieve complete remission by a series of initial chemotherapies in about 80% of AML patients, recurrence is inevitable in the majority of the patients without allogeneic transplantation. It has been reported that leukemia stem cells are resistant to

chemotherapy and that it may be an important reason why it is difficult to eradicate AML cells in the majority of patients [1]. This necessitates the development of novel therapies that have different antileukemic mechanisms. Immunotherapy may meet the requirement owing to its antileukemic mechanisms different from those of chemotherapy. Therefore, after reducing tumor burden by chemotherapy, immunotherapy is expected to be a suitable treatment modality to eliminate minimally residual leukemic cells resistant to chemotherapy.

2.2. Treatment of Elderly Patients with AML. AML most often occurs in elderly people. It has been reported that 2-year overall survival rate of elderly patients with AML is only 6% [2]. The main reasons of this poor outcome are that AML in elderly patients is often more resistant to chemotherapy than that in younger patients and that elderly patients are intolerable to intensive chemotherapy and allogeneic transplantation. The major advantage of immunotherapy is mild adverse events, and this makes immunotherapy a suitable treatment option for elderly patients.

3. AML-Associated Antigens

Recent studies have identified several promising AML antigens suitable for targets of immunotherapy.

Wilms' tumor 1 (WT1) is one of the most promising AML-associated antigens. It was originally reported that HLA-A*2402- [3] and HLA-A*0201- [4] restricted WT1 peptides induce cytotoxic T lymphocytes (CTLs) that kill WT1-expressing leukemic cells but not normal progenitor cells. WT1 is a transcription factor that plays an important role in leukemogenesis [5], and thus it is less probable that the expression of WT1 is lost. Notably, it has been repeatedly reported that immune responses against WT1 are naturally elicited in cancer patients [6–10], indicating that WT1 protein is immunogenic. These properties render WT1 highly attractive as a tumor antigen.

Proteinase 3 is a myeloid cell-restricted serine protease abundantly expressed in azurophilic granules and is another promising myeloid leukemia-associated antigen. It was originally reported that HLA-A*0201-restricted proteinase 3 peptides induce CTLs that preferentially kill myeloid leukemia cells compared to normal marrow cells [11]. Proteinase 3 has also been shown to be immunogenic, as proteinase-3-specific CTLs are induced in a substantial fraction of myeloid leukemia patients in vivo [8, 9, 12].

Other than WT1 and proteinase 3, the receptor for hyaluronic-acid-mediated motility (RHAMM/CD168) [13], human telomerase reverse transcriptase (hTERT) [14], preferentially expressed antigen in melanoma (PRAME) [15, 16], and Aurora-A [17] have been reported as potentially useful AML-associated antigens. Notably, WT1 [18] and Aurora-A [17] are reported to be expressed in leukemia stem cells and may thus be suitable targets to eradicate AML.

4. Methods of Antigen-Specific Immunotherapy for AML: Active Immunization

Antigen-specific immunotherapies can be largely divided into two categories: active immunization and adoptive T cell therapy. In active immunization, tumor antigens are injected in order to provoke antigen-specific immune responses in vivo. To do so, there are mainly the following three ways reported for AML: peptide vaccines, granulocyte-macrophage colony-stimulating-factor- (GM-CSF) secreting tumor vaccines, and dendritic cell (DC) vaccines.

4.1. Peptide Vaccines. Peptides in combination with an appropriate adjuvant are injected to stimulate CD8+ CTLs specific to the MHC-class-I-restricted peptides. WT1 peptide vaccines have been actively pursued. Oka et al. have first reported a clinical trial of HLA-A*2402-restricted WT1 peptide vaccination for malignancies including 12 AML patients [19]. Among 8 patients with evaluable disease, 5 patients achieved decreases in their AML. Notably, in two myelodysplastic syndrome (MDS) patients, numbers of leukocytes in peripheral blood, the majority of which was likely to derive from MDS clones, precipitously decreased after the first administration of the WT1 peptide [20]. This implies remarkable antitumor immune responses in these patients.

Keilholz et al. reported a clinical trial of HLA-A*0201-restricted WT1 peptide vaccination for 19 AML or MDS patients, most of whom had large tumor burden [21]. They observed clinical benefit, that is, stable disease or better, in 14 patients and increases in WT1 tetramer+ T cells in blood in 8 patients. Intriguingly, 4 patients had clinical benefit after initial progression, illustrating the importance of evaluating clinical responses to tumor vaccines at later time points even in the presence of initial progression.

Rezvani et al. reported a clinical trial of combined administration of HLA-A*0201-restricted WT1 and proteinase 3 peptides to 8 patients with myeloid malignancies [22]. Immune responses to both WT1 and proteinase 3 were detected after a single vaccination in all the patients, suggesting expansion of preexisting memory CD8+ T cells. However, the responses were short-lived and became undetectable after 4 weeks, indicating the necessity of repetitive boost injection.

Maslak et al. reported a clinical trial of a novel combination of WT1 peptide vaccination for 9 AML patients [23]. They used a mixture of 4 peptides; one is an HLA-A*0201-restricted heteroclitic peptide that has higher affinity to the HLA class I molecule than a native peptide, and three are long peptides that bind to multiple HLA-DRB1 haplotypes [24]. Interestingly, one of the long peptides embeds the HLA-A*0201-restricted heteroclitic peptide in it. The combination of peptides has three potential advantages. First, the heteroclitic peptide is expected to stimulate low avidity tumor-specific CD8+ T cells that preferentially remain in cancer patients. Second, the MHC-class-II-binding peptides can exploit CD4+ T cell help that is required to induce a robust memory CD8+ T cell response. Third, it has been reported that a long peptide containing an MHC-class-I-restricted peptide can be preferentially targeted to professional antigen-presenting DCs that are capable of presenting the embedded MHC-class-I-restricted peptide for a long period [25]. This may avoid suboptimal stimulation of CD8+ T cells resulting from administration of a short peptide that is nonselectively

presented by nonprofessional antigen-presenting cells [26]. It is difficult to judge whether this theoretically advantageous strategy leads to a better clinical outcome from the small scale of the study. However, the authors observed the induction of immune responses in 8 out of 9 patients, warranting further study in a larger clinical trial.

Schmitt et al. reported a clinical trial of the HLA-A2-restricted RHAMM peptide vaccination in patients with AML, MDS, and multiple myeloma [27]. In 7 of 10 patients, RHAMM-specific immune responses were detected. Three of 6 patients with myeloid disorders (1/3 AML, 2/3 MDS) achieved clinical responses. This study indicates that RHAMM constitutes a promising target for immunotherapy of AML.

Collectively, these clinical trials of peptide vaccines have established safety and immunogenicity of this modality. Efforts to improve clinical efficacy by combining with superior adjuvants or with other therapeutic modalities will increase the potential of peptide vaccines for AML.

4.2. GM-CSF-Secreting Tumor Vaccines. Random mutations in tumor cells are expected to generate many individually specific antigens that may induce multivalent antitumor immune responses of both CD4+ and CD8+ T cells. Thus, the whole autologous tumor cell vaccination is a viable option as long as a sufficient number of tumor cells are harvested in advance.

A mixture of killed autologous leukemia cells and a GM-CSF gene-transduced K562 leukemia cell line was used for vaccination in combination with primed T cells after autologous stem cell transplantation for 54 patients with AML [28]. Leukemic cells are expected to be incorporated into DCs activated by GM-CSF in vivo, and the DCs stimulate antigen-specific T cells. Induction of delayed-type hypersensitivity reactions to autologous tumor cells was associated with 3-year relapse free survival, suggesting a correlation between an immune response and a clinical outcome.

Autologous leukemia cells transduced with GM-CSF were administered after allogeneic transplantation to 28 patients with AML or high-risk MDS [29]. Vaccination elicited local and systemic immune responses despite the administration of a calcineurin inhibitor as prophylaxis against GVHD. Whereas the incidence of GVHD did not increase by vaccination, 9 of 10 patients achieved durable complete remission. Thus, this immunotherapy may potentiate GVL reaction without causing GVHD.

4.3. DC Vaccines. DCs generated ex vivo from monocytes or CD34+ progenitor cells are modified to present tumor antigens and are injected. It has also been reported that AML cells can be differentiated into DCs and they can be injected.

In the first DC vaccination for AML, Fujii et al. used CD34+ progenitor cell-derived DCs pulsed with autologous leukemic cells in combination with primed T cells for 4 relapsed patients after allogeneic stem cell transplantation [30]. This method induced tumor-specific immune responses. However, most of the later studies used monocytes as a source of DCs, mainly because it is technically easier to obtain DCs for vaccination from monocytes than CD34+ progenitor cells.

Clinical trials of DC-based immunotherapy for AML using leukemic cell-derived DCs have also been reported [31, 32]. However, the generation of leukemic cell-derived DCs was feasible only in a limited number of patients, and, even in patients with successful generation and vaccinations of leukemic cell-derived DCs, the DC vaccinations could not induce clinically relevant immune responses [32]. This may be due to lower immunostimulatory activity of leukemic cell-derived DCs compared with monocyte-derived DCs (MoDCs) [33], providing a rationale for the use of MoDCs in immunotherapy for AML.

Lee et al. reported the first study of MoDC-based immunotherapy for 2 AML patients with relapse after autologous peripheral blood stem cell transplantation [34]. Although immune responses were induced, the diseases progressed possibly because of high tumor burden before vaccination. In contrast, Van Tendeloo et al. recently reported immunotherapy for AML, 8 patients at complete remission and 2 at partial remission [35]. MoDCs transfected with WT1 mRNA were administered, and molecular remission was achieved in 4 patients including the 2 patients of partial remission. Clinical responses were correlated with increases in WT1-specific CD8+ T cells. This study indicates that vaccination with WT1 mRNA-loaded MoDCs as a postremission treatment may prevent full relapse.

We recently reported two clinical studies of MoDC-based immunotherapy for AML at morphologic complete remission in elderly patients. In one study, we administered MoDCs that engulfed autologous apoptotic leukemic cells to 4 patients [36]. We observed immune responses in 2 patients who exhibited disease stabilization. WT1- and hTERT-specific CD8+ T cell responses were observed in an HLA-A*2402-positive patient, indicating cross-priming in vivo. In another study, we administered MoDCs pulsed with an HLA-A*2402-restricted modified WT1 peptide that has higher affinity to the HLA molecule than the natural peptide to 3 patients [37]. We observed immune responses in 2 patients who exhibited transient disease stabilization. Notably, CD8+ T cells reactive to the WT1 natural peptide but not to the modified peptide persisted after terminating vaccination, implying that the natural peptide-reactive T cells survived due to stimulation by endogenous cognate antigens.

Collectively, these studies indicate that MoDC-based immunotherapy is immunogenic even in elderly patients with AML after remission-inducing chemotherapy and warrant further study of this strategy.

5. Methods of Antigen-Specific Immunotherapy for AML: Adoptive T Cell Therapy

Active immunization relies on immune competence of cancer patients. However, tumor antigen-specific T cells may be nonfunctional or deleted in the presence of tumor cells in vivo in cancer patients [38]. In addition, chemotherapy

and immunosuppressive factors from tumor cells may undermine antitumor immunity in cancer patients [39]. Based on these ideas, adoptive transfer of tumor-specific T cells is actively pursued.

Tumor-specific adoptive T cell therapy was initially developed by expanding tumor-infiltrating lymphocytes from melanoma lesions in vitro [40]. However, complicated procedures and difficulty in timely preparation of a sufficient number of cytotoxic T lymphocytes (CTLs) preclude generalization of this strategy. To overcome these drawbacks, adoptive T cell therapies using genetically engineered T cells have recently been prevailing. There are two measures: (i) CD8+ T cells transduced with genes encoding T cell receptor (TCR) that recognizes the complex of a tumor peptide and a particular MHC class I molecule and (ii) T cells transfected with genes encoding chimeric antigen receptor (CAR) that is composed of antibody and cytoplasmic domain of the CD3 molecule.

5.1. Adoptive Transfer of T Cells with Transgenic TCR. The first clinical trial of TCR-transduced T cell transfer was performed to advanced melanoma patients by Rosenberg's group, using HLA-A*0201-restricted MART-1, gp100, NY-ESO-1, and p53 as targeted antigens [41]. The transduced T cells were administered after lymphodepleting regimen of fludarabine and cyclophosphamide. Two out of 17 patients achieved partial remission. The absence of therapeutic effects in most cases may be related to the failure of the infused cells to accumulate into the tumor or to exert their effector function in the immunosuppressive tumor microenvironment.

Subsequently, the same group reported a clinical trial using high avidity TCR against HLA-A*0201-restricted MART-1 and gp100 peptides [42]. Objective cancer regressions were observed in 30% of patients. Gene-engineered cells persisted at high levels in the blood of all patients 1 month after treatment. However, patients exhibited destruction of normal melanocytes in the skin, eye, and ear. In another study by the same group, a retrovirus encoding the high avidity murine CEA-reactive TCR was used to transduce peripheral blood lymphocytes from 3 HLA-A*0201+ patients with metastatic colorectal cancer [43]. All patients experienced profound decreases in serum CEA levels. However, a severe transient inflammatory colitis was induced in all 3 patients. These studies indicate excellent antitumor activity as well as destructive power of highly avid T cells against normal tissues, suggesting the importance of careful assessment of possible damage to normal tissues that share the target antigen with tumor cells.

These promising results of adoptive transfer of TCR-transduced T cells for solid tumors pave the way for its application to hematological malignancies. Two groups reported mouse models of adoptive transfer of T cells with WT1-specific TCR genes [44, 45]. Both of the groups recently reported in vivo therapies for AML using mouse xenograft models transferred with WT1 TCR-transduced T cells [46, 47].

In the T cells transduced with a new TCR gene, their original TCRs are still functional, and thus mispairing of endogenous and introduced TCR chains occurs. This decreases the expression level of introduced TCR, resulting in reduced antitumor activity [48]. In addition, studies in murine models with TCR gene transfer have shown that the mispairing may generate neoreactivity against autoantigens, resulting in GVHD [49]. Ochi et al. circumvented the mispairing problem in an elegant way by developing a novel retroviral vector system for TCR gene transfer that can selectively express target antigen-specific TCR while expression of intrinsic TCRs is suppressed by built-in siRNAs [47, 50]. In a mouse xenograft model, adoptively transferred WT1-siTCR gene-transduced T cells exerted distinct anti-leukemia efficacy, but did not inhibit human hematopoiesis [47]. This is a promising report heading for a clinical trial to treat AML using WT1-TCR T cell transfer.

5.2. Adoptive Transfer of T Cells with Transgenic CAR. A CAR contains an extracellular antigen-binding domain, a transmembrane region, and a signaling endodomain. The extracellular domain is typically a single chain variable fragment (scFv) derived from a tumor-specific monoclonal antibody. There are two advantages of using an antibody-derived domain for antigen recognition. First, antibodies are not dependent on MHC presentation. Second, antibodies bind antigens with much greater affinity than TCRs, permitting the formation of a more stable immunological synapse.

CARs can be grouped into three generations with progressively increasing costimulatory activity. These differ primarily in the structure of the signaling endodomain. First-generation CARs contain a single signaling unit derived from the CD3 chain alone, which transmits a signal inadequate to fully activate T cells. In second-generation CARs, the CD28 intracellular domain is inserted proximal to the CD3 endodomain to enhance the stimulatory effects of the CAR. This encouraged further addition of other signaling sequences from costimulatory molecules such as 4-1BB and OX40 in third-generation CARs. A complete response was observed in a patient with follicular lymphoma who received T cells transduced with a second-generation anti-CD19 CAR [51]. However, the supraphysiological signal transmitted by second- and third-generation CARs is also a source of concern. In fact, 2 deaths in cancer patients treated with CAR T cells occurred apparently due to cytokine storm: one patient with colon cancer treated with ERBB2-specific CAR [52] and another with chronic lymphocytic leukemia treated with CD19-specific CAR [53]. Although these serious adverse events indeed suggest highly active antitumor effects of CAR, modification to decrease T cell doses and to split infusions will be important to reduce such risk.

The carbohydrate antigen Lewis^Y is expressed in about 50% of multiple myeloma and AML cases. Lewis^Y CAR-transduced T cells delayed growth of myeloma xenografts in NOD/SCID mice [54]. This paper indicates that Lewis^Y CAR T cell transfer is a promising therapy for myeloma and AML.

6. Conclusions and Future Prospects

Advances in immunology and identification of promising leukemia-associated antigens are making it possible to

develop truly effective immunotherapies for AML. Fortunately, AML is relatively more chemosensitive than most solid tumors, and thus it is possible to reduce a tumor burden by chemotherapy in the majority of patients. Thereafter, immunotherapy will play a complementary role in eradicating minimal residual diseases, which contain chemoresistant leukemia stem cells. Thus, leukemia-associated antigens expressed in leukemia stem cells will be important to achieve cure.

Recent concerns are immunosuppressive factors expressed by tumor cells or built in the immune system, which curtail antitumor immunity. Universal immunosuppressive factors built in the immune system, such as CTLA-4 [55, 56], PD-1 [57, 58], and regulatory T cells [59–61], are widely applicable targets in combination with antitumor vaccination. However, CTLA-4 blockade caused autoimmune manifestations in considerable fractions of patients [55, 56], which is anticipated from the role of CTLA-4 in maintaining immune homeostasis. Such adverse events, in addition to the autoimmunity [42, 43] and life-threatening cytokine storm [52, 53] observed in the adoptive T cell transfer, indicate that pursuing effectiveness of tumor immunotherapy inherently raises the possibility of harmful immune reactions, if the target antigen is shared by tumor and normal cells or the tumor burden is high. Balance between effectiveness and adverse events will thus become a main issue in the era of advanced immunotherapy. Still, "relative" tumor specificity of immunotherapy, at least, compared to other modalities of cancer therapy will make immunotherapy an indispensable facet of antitumor armamentarium.

Furthermore, epigenetic therapies with DNA methyltransferase and histone deacetylase inhibitors are prevailing as novel therapies for myeloid malignancies. Notably, recent studies have shown that epigenetic modification upregulates the expression of cancer testis antigens in AML and induces CTL responses [62, 63]. This raises possibilities for reasonable combinations of therapies targeting molecular oncogenic pathways and immunotherapies. Such prospects will collectively open an exciting new era of AML therapies in the near future.

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Research Article

Systemic Administration of CpG Oligodeoxynucleotide and Levamisole as Adjuvants for Gene-Gun-Delivered Antitumor DNA Vaccines

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DNA vaccines showed great promise in preclinical models of infectious and malignant diseases, but their potency was insufficient in clinical trials and is needed to be improved. In this study, we tested systemic administration of two conventional adjuvants, synthetic oligodeoxynucleotide carrying immunostimulatory CpG motifs (CpG-ODN) and levamisole (LMS), and evaluated their effect on immune reactions induced by DNA vaccines delivered by a gene gun. DNA vaccination was directed either against the E7 oncoprotein of human papillomavirus type 16 or against the BCR-ABL1 oncoprotein characteristic for chronic myeloid leukemia. High doses of both adjuvants reduced activation of mouse splenic $CD8^+$ T lymphocytes, but the overall antitumor effect was enhanced in both tumor models. High-dose CpG-ODN exhibited a superior adjuvant effect in comparison with any combination of CpG-ODN with LMS. In summary, our results demonstrate the benefit of combined therapy with gene-gun-delivered antitumor DNA vaccines and systemic administration of CpG-ODN or LMS.

1. Introduction

After pioneering studies showing the expression of protein antigens from plasmid DNA and the ability of these antigens to induce both humoral and cell-mediated immunity in the early 1990s [1–3], DNA vaccines against some infectious diseases and also malignant tumors were quickly developed and successfully tested in animal models. However, the efficacy of DNA immunization in initial clinical trials was disappointing [4].

Immune reactions induced by DNA vaccines can be enhanced by adjuvants that are classified into two groups by Sasaki et al. [5]—genetic and conventional. While genetic adjuvants are plasmids producing cytokines, chemokines or other immunomodulatory molecules, conventional adjuvants are chemical compounds increasing or modulating immune responses. As genetic adjuvants are of the same nature as DNA vaccines, they can be easily codelivered in any method of DNA vaccine administration. However, conventional adjuvants can be mixed and codelivered with DNA vaccines injected as a solution, but their codelivery with DNA vaccines administered via a gene gun is limited by the mode of application. Only local application of the conventional adjuvant imiquimod was more widely tested in combination with gene-gun delivery of plasmid DNA [6, 7].

Of five conventional (chemical) adjuvants tested after addition to an intramuscular DNA vaccine, levamisole (LMS), a synthetic phenylimidazolthiazole, induced the strongest Th1 immune reactions [8]. The high immunostimulatory activity of LMS in DNA vaccination was confirmed in subsequent studies [9, 10]. This compound developed as an anthelmintic drug in the 1960s is also recommended, in combination with 5-fluorouracil, as adjuvant chemotherapy for colon cancer [11, 12].

Moreover, the effect of DNA vaccination is supported by immunostimulatory unmethylated CpG motifs that can be

either carried by an immunization plasmid itself or delivered on synthetic oligodeoxynucleotides (ODNs) [13]. Demonstrated in animal models, the benefit of ODNs carrying CpG motifs (CpG-ODN) after addition to various types of vaccines was evaluated in clinical trials [14, 15]. However, systemic administration of ODNs caused suppression of splenic cytotoxic T lymphocytes (CTLs) in mice, which raised concerns for the usability of CpG-ODN in antitumor therapy. This effect was associated with enhanced production of indoleamine 2,3-dioxygenase (IDO) by splenic $CD19^+$ dendritic cells (DCs) [16, 17]. Systemic injection of CpG-ODN also diminished cross-presentation of antigens by DCs [18]. On the other hand, repeated systemic administration of high doses of CpG-ODN induced immune-mediated protection from acute lymphoblastic leukemia [19].

In this study, we evaluated the influence of systemic administration of LMS and CpG-ODN on the activation of mouse splenic CTLs by gene-gun DNA vaccination and on the antitumor effect elicited in models of chronic myeloid leukemia (CML) and human-papillomavirus- (HPV-) induced tumors. These adjuvants were compared for potency and combined treatment was examined as well.

2. Materials and Methods

2.1. Plasmids. The plasmids pBSC [20], pBSC/bcr-abl [21], pBSC/E7GGG.GUS [22], and pBSC/EGGG.LAMP [23] were used for immunization. The plasmid pBSC/bcr-abl produces the protein BCR-ABL1 (p210) from the fusion gene generated by the b3a2 chromosomal translocation t(9; 22) in a CML patient. The fusion gene E7GGG.GUS consists of the mutated HPV16 E7 gene (E7GGG) containing three point mutations resulting in substitutions D21G, C24G, and E26G in the Rb-binding site [20] and the gene encoding *E. coli* β-glucuronidase (GUS). In the E7GGG.LAMP gene, E7GGG was fused with two signal sequences of lysosome-associated membrane protein 1 (LAMP-1).

2.2. Cell Lines. TC-1 cells, kindly provided by T. C. Wu (Johns Hopkins University, Baltimore, Md), were prepared by the transformation of C57BL/6 mouse primary lung cells with the HPV16 E6/E7 oncogenes and the activated human *H-ras* gene [24]. TC-1 cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM; PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum (FCS; PAA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

12B1 cells producing the BCR-ABL1 (b3a2) protein [25] were obtained through the courtesy of E. Katsanis (University of Arizona, Tucson, Ariz). They were derived by transformation of BALB/c mouse primary bone marrow cells with a retrovirus-derived vector carrying the BCR-ABL1 fusion gene. 12B1 cells were passaged in RPMI-1640 medium (Sigma-Aldrich, St. Louis, Mont) supplemented with 10% FCS, 1 mM pyruvate, 50 mM 2-mercaptoethanol, L-glutamine, and antibiotics.

2.3. Mice. Six- to eight-week-old female C57BL/6 ($H\text{-}2^b$) or BALB/c mice ($H\text{-}2^d$; Charles River, Germany) were used in immunization experiments. Animals were maintained under standard conditions at the Center for Experimental Biomodels, Charles University, Prague.

2.4. Immunization Experiments. Plasmid DNA was coated onto 1 μm gold particles (Bio-Rad, Hercules, Calif) as described previously [20]. Mice were immunized with plasmids by a gene gun (Bio-Rad) at a discharge pressure of 400 psi into the shaven skin of the abdomen. Each immunization consisted of one or two shots delivering 1 or 2 μg of plasmid DNA.

For *in vitro* examination of immune reactions, C57BL/6 mice (three per group) were immunized with two 1 μg doses of the E7GGG.GUS plasmid given one week apart. In therapeutic immunization experiments, C57BL/6 or BALB/c mice (six per group) were first s.c. administered 3×10^4 TC-1 or 5×10^3 12B1 cells suspended in 150 μL or 200 μL PBS, respectively, into the back and then vaccinated with pBSC/E7GGG.LAMP (1 μg doses three and ten days after cell inoculation) or pBSC/bcr-abl (2 μg doses three, six, and ten days after cell inoculation), respectively. The empty pBSC plasmid was used as a negative control. Tumor cells were administered under anesthesia with intraperitoneal etomidate (0.5 mg/mouse; Janssen Pharmaceutica, Beerse, Belgium). Tumor growth was monitored twice a week, and tumor size was calculated from three perpendicular measurements using the formula $(\pi/6)$ $(a \times b \times c)$. Mice were sacrificed when tumor volume reached 1 cm^3 or two months after cell inoculation.

The adjuvants phosphorothioate-stabilized oligodeoxynucleotide ODN1826 carrying CpG immunostimulatory motifs (TCCATGACGTTCCTGACGTT; Generi Biotech, Hradec Kralove, Czech Republic) and LMS (Sigma-Aldrich) dissolved in 200 μL PBS were i.p. injected on the days of DNA vaccination.

2.5. Tetramer Staining. A week after immunization with pBSC/E7GGG.GUS, tetramer staining was performed as described previously [26]. In brief, lymphocyte bulk cultures were prepared from splenocytes of three immunized animals and restimulated with the HPV16 $E7_{49-57}$ peptide (RAHYNIVTF) for 6 days. After incubation with anti-mouse CD16/CD32 antibody (Fc-block; BD Biosciences, San Diego, Calif), lymphocytes were stained with a mixture of $H\text{-}2D^b/E7_{49-57}$-PE tetramers (Sanquin, Amsterdam, The Netherlands) and anti-mouse CD8a-FITC antibody (BD Biosciences). The stained cells were measured on a Coulter Epics XL flow cytometer (Coulter, Miami, Fla) and analyzed by FlowJo 7.2.2 software (TreeStar, Ashland, Ore).

2.6. Statistical Analysis. Tumor growth was evaluated by two-way analysis of variance, tumor formation by log-rank test, and the expansion of E7-specific splenocytes in tetramer assay by Student's t test. Results were considered significantly different if $P < 0.05$. Calculations were performed using

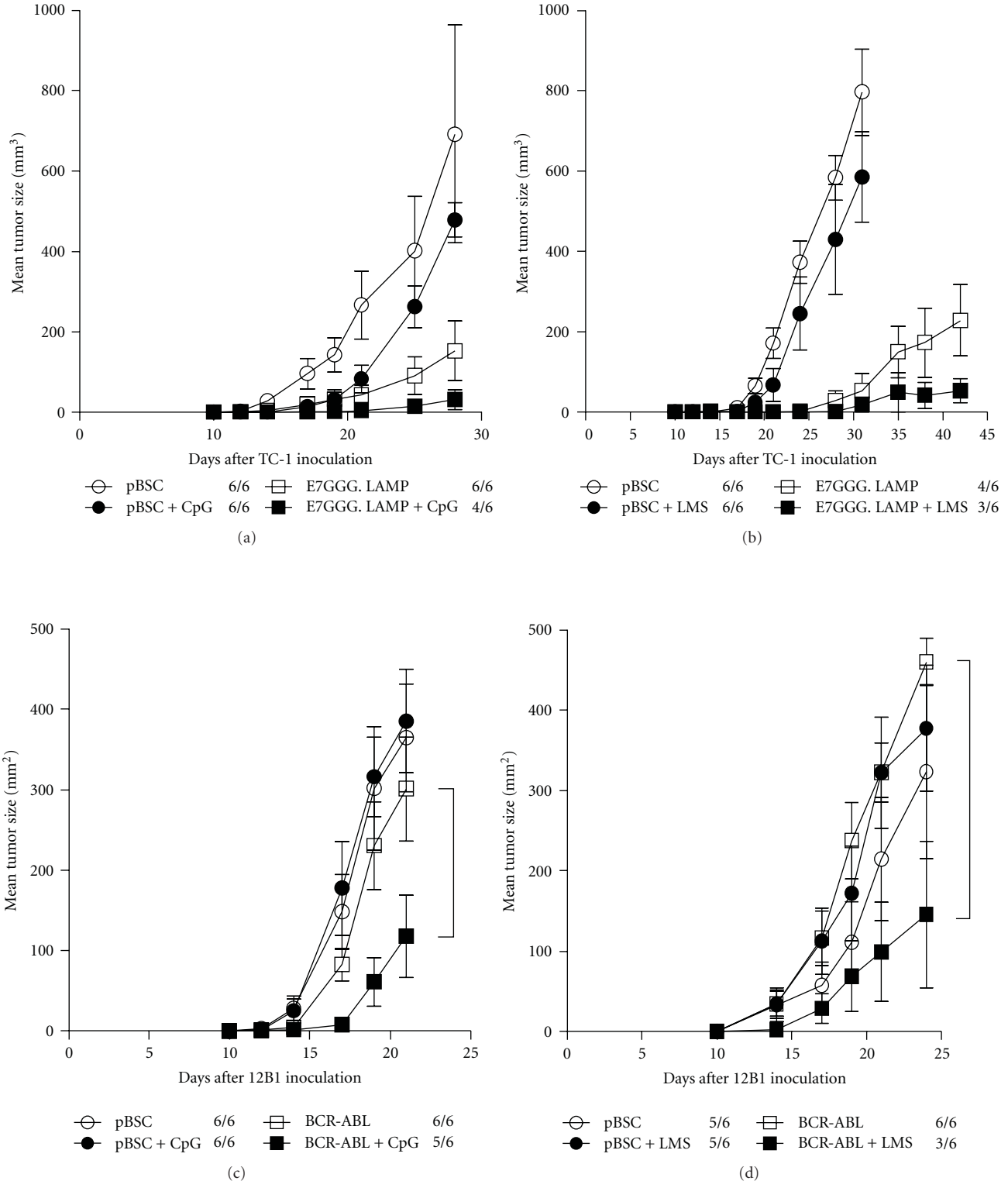

Figure 1: Antitumor effect of systemic administration of high-dose adjuvants. C57BL/6 or BALB/c mice ($n = 6$) were s.c. inoculated with 3×10^4 TC-1 (a, b) or 5×10^3 12B1 cells (c, d) and immunized by a gene gun with 1 μg of pBSC/E7GGG.LAMP three and ten days later or with 2 μg of pBSC/bcr-abl three, six, and ten days later, respectively. The pBSC plasmid was used as a negative control. CpG-ODN (50 μg; a, c) or LMS (200 μg; b, d) was i.p. injected on the days of DNA vaccination. No. of mice with a tumor/no. of mice in the group is indicated. Bars: ±SD; $^*P < 0.05$; $^{**}P < 0.01$.

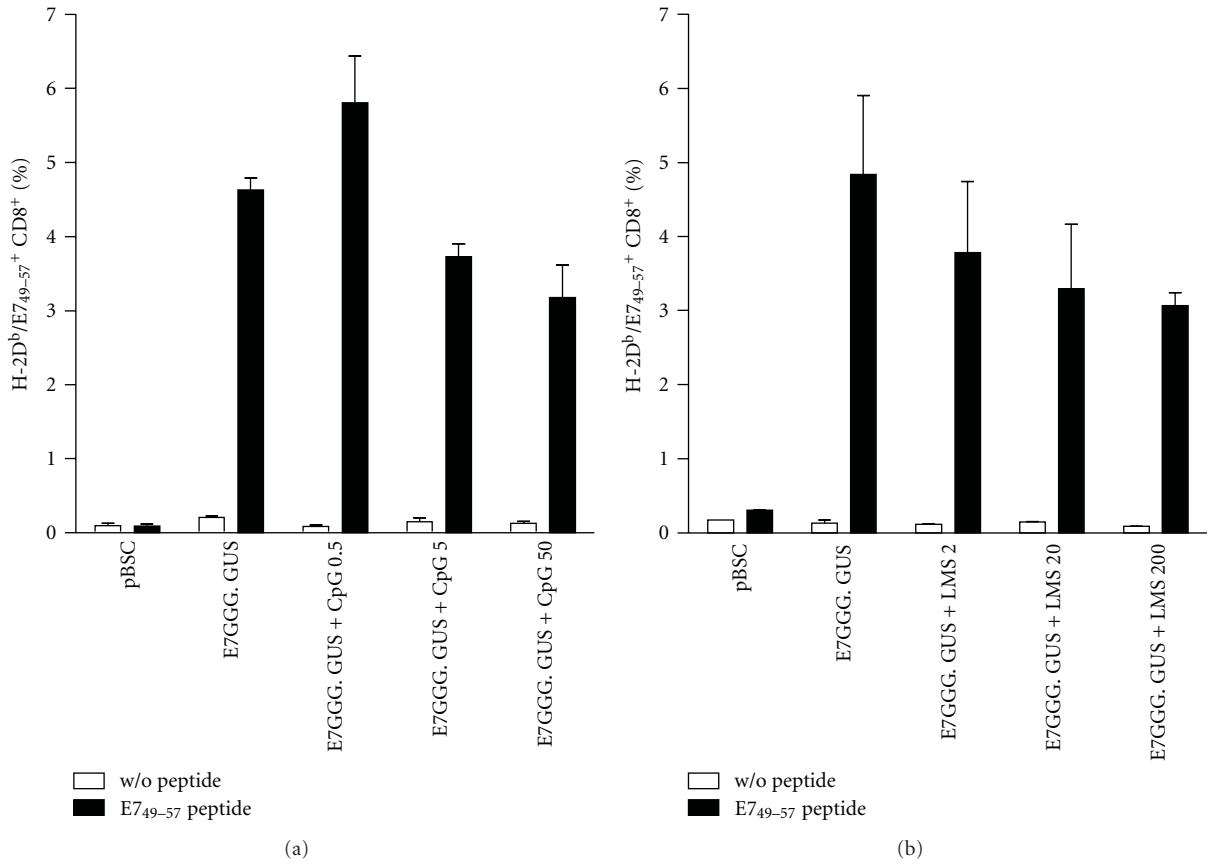

Figure 2: Detection of E7-specific splenic CTLs after DNA vaccination combined with systemic administration of adjuvants. C57BL/6 mice ($n = 3$) were twice immunized at a 1-week interval with 1 μg of pBSC or pBSC/E7GGG. GUS by a gene gun and i.p. injected with indicated doses of CpG-ODN (a) or LMS (b). One week after the second immunization, lymphocyte bulk cultures were prepared from splenocytes, restimulated with the RAHYNIVTF peptide for 6 days, and stained with a mixture of H-2D^b/E7$_{49–57}$-PE tetramers and anti-mouse CD8a-FITC antibody. Control lymphocytes were cultured without the peptide. Columns: mean of duplicate samples; bars: ±SD; $^*P < 0.05$ (the comparison with the E7GGG.GUS group).

GraphPad Prism 5.0 software (GraphPad Software, San Diego, Calif).

3. Results

3.1. Systemic Administration of High-Dose CpG-ODN or LMS Supports Antitumor Effect of Gene-Gun DNA Vaccination. We tested the influence of systemic application of CpG or LMS on antitumor effect induced by DNA vaccines delivered with a gene gun in two mouse tumor models: TC-1 cells producing the HPV16 E7 oncoprotein and 12B1 cells producing the BCR-ABL1 fusion protein injected s.c. into C57BL/6 and BALB/c mice, respectively. Because of high efficacy of immunization against the E7 antigen, we used the pBSC/E7GGG.LAMP plasmid that is less immunogenic than pBSC/E7GGG.GUS and applied only two 1 μg doses. The plasmid pBSC/bcr-abl is potent in preventive immunization against 12B1 cells [27], but its efficacy in therapeutic immunization is low. Therefore, vaccination against 12B1 cells consisted of three 2 μg doses.

For initial experiments, we chose relatively high doses of adjuvants: 50 μg of CpG-ODN and 200 μg of LMS. Both adjuvants reduced the growth of TC-1-induced tumors in animals either immunized or nonimmunized against the E7 antigen, but this effect was nonsignificant (Figures 1(a) and 1(b)). However, while adjuvants alone did not affect 12B1-induced tumors, they significantly reduced tumor growth after combination with vaccination (CpG-ODN: $P = 0.027$, LMS: $P = 0.008$; Figures 1(c) and 1(d)). Moreover, in both tumor models, administration of adjuvants to immunized mice resulted in inhibition of tumor formation in a portion of animals. This effect was significant for combination of pBSC/bcr-abl immunization and LMS administration ($P = 0.019$).

3.2. Systemic Administration of High Doses of CpG-ODN or LMS Reduces the Stimulation of Splenic CTLs by Gene-Gun DNA Vaccination. As systemic inoculation of 50 μg of CpG-ODN has been reported to reduce the CTL activity induced by immunization [16, 17], we evaluated this effect

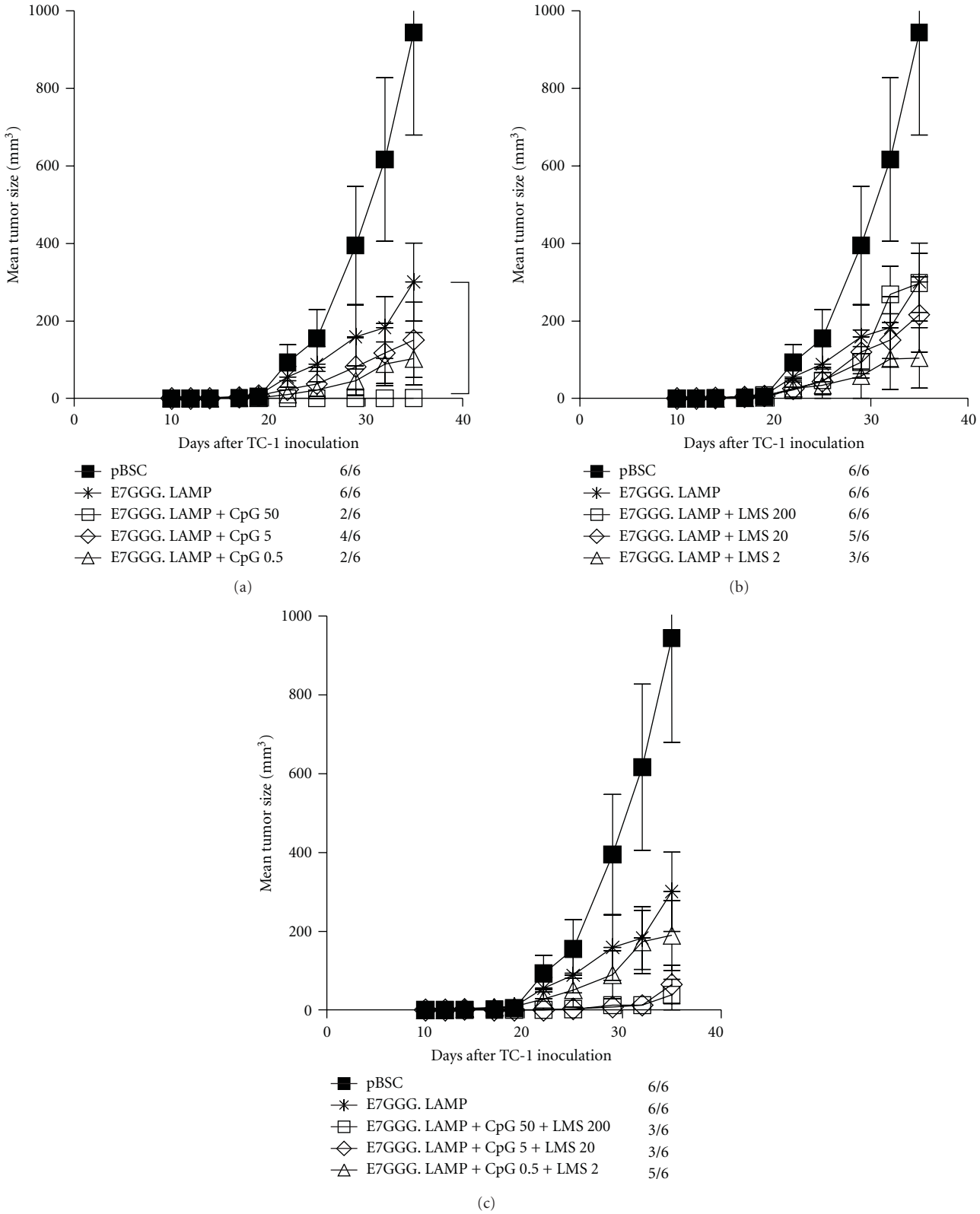

Figure 3: Antitumor effect of systemic administration of single adjuvants or their combinations. C57BL/6 mice ($n = 6$) were s.c. inoculated with 3×10^4 TC-1 cells and immunized by a gene gun with 1 μg of pBSC/E7GGG.LAMP three and ten days later. The pBSC plasmid was used as a negative control. CpG-ODN (a), LMS (b), or their combinations (c) were i.p. injected at indicated doses on the days of DNA vaccination. The graphs (a), (b), and (c) were constructed from the results of the same experiment. No. of mice with a tumor/no. of mice in the group is indicated. Bars: ±SD; $^*P < 0.05$.

for CpG-ODN and LMS after vaccination of C57BL/6 mice with the pBSC/E7GGG.GUS plasmid. The stimulation of splenic CTLs specific for the H-2D^b E7 epitope was measured after the addition of CpG-ODN or LMS at doses used for enhancement of the antitumor effect of the DNA vaccines (i.e., 50 μg and 200 μg, resp.) and two lower doses (5 and 0.5 μg for CpG-ODN and 20 and 2 μg for LMS). Both adjuvants exhibited reduction of E7-specific CD8$^+$ T lymphocytes in the spleens (Figure 2). The extent of this inhibition was similar for the highest ($P < 0.05$) and medium doses of CpG-ODN and LMS, but, while the lowest dose of LMS was still inhibiting CTL response (Figure 2(b)), that of CpG-ODN moderately increased it (Figure 2(a)).

3.3. Combination of CpG-ODN and LMS Does Not Outperform CpG-ODN Alone in Supporting Antitumor Effect of Gene-Gun DNA Vaccination. Showing lower inhibition of CTLs with lower doses of adjuvants, we next compared the antitumor effects of the three doses in the model of TC-1-induced tumors. Simultaneously, we tested combinations of both adjuvants. In control mice immunized with the pBSC/E7GGG.LAMP plasmid, tumor growth was markedly reduced in comparison with pBSC-treated mice, but tumors developed in all animals (Figure 3). For CpG-ODN, the highest dose of the adjuvant (50 μg) most efficiently supported antitumor immunity elicited by DNA vaccination—tumor formation was inhibited in four out of six mice ($P = 0.005$) and tumor growth was significantly reduced ($P = 0.033$; Figure 3(a)). Conversely, the administration of the lowest dose of LMS (2 μg) resulted in the lowest tumor rate (3/6, $P = 0.034$; Figure 3(b)). Combinations of high and medium doses of adjuvants provided the best antitumor effects (tumor rate 3/6, $P = 0.020$ and $P = 0.041$, resp.; Figure 3(c)), but none of them outperformed the high-dose CpG-ODN in terms of potency.

4. Discussion

Successful examination of DNA vaccines in animals resulted in license acquisition by several veterinary vaccines directed against both infectious and malignant diseases. The evaluation of DNA vaccines in clinical trials showed that these vaccines were well tolerated and safe, but their immunogenicity was unexpectedly lower than in preclinical models. In recent years, progress in enhancing the efficacy of DNA vaccination in humans has been achieved mainly thanks to the improvement of physical delivery methods, with muscle electroporation and particle bombardment of the skin being currently predominant [28].

Utilization of adjuvants that is crucial for a high efficacy of protein and peptide vaccines is still in its infancy in DNA immunization. Their introduction into clinical immunization with DNA vaccines could be another step in the enhancement of DNA vaccination efficacy. In this study, we tested systemic administration of two adjuvants, CpG-ODN and LMS, in combination with gene-gun DNA immunization and evaluated adjuvant-mediated impact on the antitumor effect induced by DNA vaccines.

At high doses, both adjuvants reduced activation of specific splenic CTLs, but, overall, they enhanced the antitumor potency of DNA vaccination. Inhibition of splenic CTLs by CpG-ODN has already been reported, and increased expression of IDO by splenic CD19$^+$ DCs has been identified as a key factor in this process [16, 17]. However, CpG-ODN directly or indirectly affects other immune cells, including different types of DCs, T cells, NK cells, B cells, monocytes, and neutrophils, that can contribute to reduced tumor growth [29]. Similarly, LMS activates DCs and induces their maturation, which leads to stimulation of CTLs [30]. Thus, complex activation of the immune system by the two systemically delivered adjuvants can result in strengthened immunity in the tumor despite mild immunosuppression in the spleen.

CpG-ODN and LMS activate DCs by binding to Toll-like receptor- (TLR-) 9 and TLR-2 [30], respectively. Both adjuvants induce production of IL-12 and stimulate Th1 immune response. Our comparison showed higher potency of CpG-ODN in enhancement of antitumor effect against mouse TC-1 tumor cells. Combinations of various doses of CpG-ODN and LMS did not further increase the impact on tumor growth. However, subsets of mouse and human DCs differ in TLR-9 and TLR-2 expression [31]: while all mouse DC subsets produce both TLRs, human myeloid DCs produce only TLR-2 and plasmacytoid DCs only TLR-9. Then, in humans, the combination of CpG-ODN and LMS can be useful in antitumor treatment.

Systemic administration of CpG-ODN is well tolerated and induces Th1 immune response in humans [32]. As preclinical models demonstrated improved effect of chemotherapy after addition of CpG-ODN, clinical trials examining this combined treatment have also been launched [14]. Furthermore, recent results in mouse tumor models suggested the potential of systemic administration of CpG-ODN in the inhibition of metastasis [33] and treatment of minimal residual disease [19]. This study showed that vaccination could supplement such methods of antitumor therapy with systemic CpG-ODN delivery.

5. Conclusions

Our results demonstrate that in spite of partial inhibition of specific immunity by systemic administration of high-dose CpG-ODN or LMS, these adjuvants potentiated the antitumor effect of DNA vaccines delivered by a gene gun. CpG-ODN was more efficient than LMS, and combination of both adjuvants did not outperform CpG-ODN alone in terms of potency. To conclude, we propose a new approach to enhancing antitumor gene-gun DNA vaccination: systemic CpG-ODN delivery.

Acknowledgments

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Review Article

A Possible Anticancer Agent, Type III Interferon, Activates Cell Death Pathways and Produces Antitumor Effects

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Recently identified interleukin-28 and -29 belong to a novel type III interferon (IFN) family, which could have distinct biological properties from type I and II IFNs. Type I IFNs, IFN-α/β, have been clinically applied for treating a certain kind of malignancies for over 30 years, but a wide range of the adverse effects hampered the further clinical applications. Type III IFNs, IFN-λs, have similar signaling pathways as IFN-α/β and inhibits proliferation of tumor cells through cell cycle arrest or apoptosis. Restricted patterns of type III IFN receptor expression in contrast to ubiquitously expressed IFN-α/β receptors suggest that type III IFNs have limited cytotoxicity to normal cells and can be a possible anticancer agent. In this paper, we summarize the current knowledge on the IFN-λs-mediated tumor cell death and discuss the functional difference between type I and III IFNs.

1. Introduction

Interferons (IFNs) have been described as agents mediating antiviral responses over the years; however, further investigations are still required to clarify the biological properties and the mechanisms responsible for the functions [1]. There are 3 IFN families currently known, which have different receptor structures [2, 3]. Type I IFN family consists of IFN-α and IFN-β in human and binds a common heterodimeric receptor complex, composed of IFNAR1 and IFNAR2 [4]. Type II IFN comprises of IFN-γ, which is not homologous to type I IFNs in the structure, and binds a different receptor complex, IFNGR1 and IFNGR2 [2, 3]. The interaction of type I IFNs with the receptor complex induces activation of signal transducer and activator of transcription (STAT) family members, resulting in complex formations with different transcription factors [4]. IFN-γ shares similar but distinct signal transduction pathways compared with that of type I IFNs and has biologically different functions from type I IFNs.

Type III IFNs, the newest IFN family, has been identified as IFN-λ which includes 3 subtypes, IFN-λ1, -λ2 and -λ3, also known as interleukin-29 (IL-29), IL-28A, and IL-28B, respectively, [5, 6]. The receptor complexes have also been identified, and the interaction between the ligand and the receptors seems to activate identical signal transduction pathways as do type I IFNs. Nevertheless, type III IFNs could have different functions from the type I IFNs since type III IFNs bind a specific receptor complex with restricted expression manners.

2. Structure of Type III IFNs and the Receptor Complex

Type III IFNs are similar to IL-10 family cytokines in the structure as well as the type I IFNs [5, 6]. Type III IFNs can thereby represent a possible evolutionary linkage between the type I IFNs and the IL-10 family. All the *type III IFN* genes are clustered on chromosome 19 in human and consist of several exons, whereas the *type I IFNs* are mapped on chromosome 9 with a single exon. Murine *type III IFN* genes have also been indentified, *mIFN-λ1, -λ2 and -λ3,* but the *mIFN-λ1* gene has a stop codon, producing nonfunctional truncated protein [7]. Interestingly, sequences of bird and zebra fish *IFNs* suggest that type III IFNs may represent an ancestral IFN prototype that gave rise to intron-less type I IFNs by retroposition events and gene duplications [8–10].

All of the type III IFNs bind the same heterodimeric receptor, consisting of a newly identified subunit, IL-28Rα, and the IL-10Rβ subunit. IL-10Rβ is a subunit of the receptor complex for IL-10 and the IL-10-related cytokines such as IL-22 and IL-26 [11]. Similar to other class II cytokines receptors, IL-28Rα seems to determine the ligand binding specificity and recruit intracellular signaling molecules. IL-28Rα is also alternatively spliced to produce 2 variants receptors; one encodes a receptor with a 29-amino acids deletion in the intracytoplasmic portion and the other only encodes the ectodomain. Biological significances of the isoforms remain uncharacterized, but they could serve as a dominant negative form to inhibit the ligand binding or the signal transduction.

The type I IFN receptors are expressed in virtually all the somatic cells. In contrast, IL-28Rα expression seems to be restricted in a tissues-specific manner although IL-10R is ubiquitously expressed. The IL-28Rα transcripts are undetectable in several cell types such as fibroblastic and endothelial cells [12]. The limited expression of IL-28Rα is also shown in tumor cells, and the restricted expression of the receptor complex determines the repertoire of type III IFNs responsiveness, which may generate distinct biological functions from type I IFNs. The IL-28Rα expression levels are different even among the same cell lineages as found in melanoma cells [13], but it is uncertain that the levels are correlated with the responsibility to type III IFNs. Interestingly, the expression, which was evidenced in the majority of human melanoma specimens, was not identified in premalignant benign nevi specimens [13]. IL-28Rα can be inducible as type I IFN receptors; peripheral blood mononuclear cells negative for the IL-28Rα became positive for the expression when treated with IL-4 and granulocyte-macrophage colony-stimulating factor treatments [14].

3. Signaling Pathways

Antiviral responses are one of the major functions of IFNs, and the Toll-like receptors- (TLRs-) mediated pathways are essential in sensing of pathogens. The receptors detect most types of viruses by recognizing the nucleic acids and act as prototypical receptors to activate innate immunity. In particular, both TLR8 and TLR9 contribute to type I IFN production. Almost all of the nucleated cells response to viral infection and secrete type I IFNs, in which a number of molecules are involved including retinoic acid inducible gene-I (RIG-1) [15]. The same TLR8 and TLR9 activate type III IFNs production, and the induction mechanisms seem to be similar to those of type I IFNs [16]. Nevertheless, stimulation by either RNA or DNA viruses was less potent to produce type III IFNs compared with type I [17]. In addition, type III IFNs expressions were further augmented by IFN-α through their upregulated TLRs- and RIG-I-mediated signaling pathways. In contrast, hepatitis C virus infection induced rather *IFN-λs* but not *IFN-α* or *IFN-β* mRNA [18]. These data imply that type III IFNs cover the different range of virus infections from type I IFNs and can interact with other cytokines for antiviral activities.

Intercellular signal cascade systems are shared between type I and III IFNs (Figure 1). Both type IFNs activate Janus tyrosine kinase- (JAK-) STATs pathways. Ligation of the IFNs with respective receptors results in rapid phosphorylation and activation of the receptor-associated tyrosine kinase 2 (TYK2) and JAK1, which in turn induce phosphorylation and activation of STAT1, STAT2, STAT3, and STAT5. These activated STATs form hetero- or homodimeric structures, which are subsequently translocated to nucleus and bind to IFN-stimulated response elements (ISREs) in regulatory regions of the IFN-stimulated genes (ISGs). ISG factor 3 (ISGF3) is a transcriptional complex, composed of phosphorylated STAT1 and STAT2, and IFN-regulatory factor-9 (IRF-9) and initiates transcriptions of ISGs. Phosphorylated STATs complexes also bind the IFN-γ activation site (GAS) and start transcriptions of ISGs. A possible difference between type I and III IFNs could be prolonged activation of STAT1 and STAT2 by type III IFNs, which is accompanied by *de novo* STATs protein synthesis and delayed degradation [19]. The downstream signaling of type III IFNs itself is indistinguishable from that of type I IFNs. A microarray analysis demonstrated IFN-λ1 upregulated 60 genes, most of which belong to ISGs group and are the same as found in IFN-α stimulation [13]. For example, 2′,5′OAS and myxovirus resistance protein (MxA), both of which are involved in viral protection, are induced by type I and type III IFNs and, likewise, expression levels of major histocompatibility complex (MHC) class I molecules, being favorable for antiviral immunity, are also upregulated in type I and type III IFNs-treated cells. Several lines of studies indicated that the induction levels were lower in type III than in type I IFNs, which may be attributable to a possible difference in the activation processes between the types. These evidences also raise a question as to the biological significance of type III IFNs in host defense mechanisms.

IFN pathways have an alternative circuit besides the JAK-STATs-mediated system. The phosphatidyl-inositol-3-kinase (PI3K) and the p38 kinase pathways have a certain role in the IFN-induced signal transduction. More importantly, activation of the PI3K pathway is dependent on cell types and the p38 kinase pathway can modulate type-I-IFN-dependent responses. It is however currently unknown whether type III IFNs can activate the PI3K and the p38 kinase pathways. A recent study nevertheless showed that type III IFNs induced

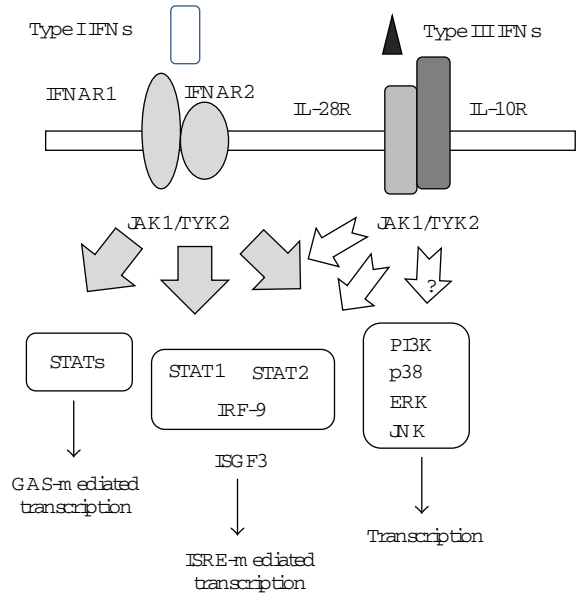

Figure 1: Signaling pathways mediated by type I and type III IFNs. Type I IFNs binding to the receptor complex induces JAK1 and TYK2 activation and phosphorylation of STAT1 and STAT2. The phosphorylated STAT1/STAT2 complex with IRF-9 forms ISGF3, which binds ISRE and initiates a number of transcriptions. Type I IFNs also activate STATs without forming ISGF3 and transactivate IFN-inducible genes through GAS elements. Additionally, type I IFNs activate the PI3K and p38 pathways to stimulate transcription of relevant genes through a number of transcription factors such as AP-1. Similarly, type III IFNs induce the JAK-STATs pathways; however, it is currently unknown whether type III IFNs activate the PI3K and p38 pathways.

the activation of the mitogen-activated protein (MAP) and both the p38 and Jun N-terminal kinase-MAP kinases were involved in the gene expression [20]. On the other hand, a different study with human melanoma cells implied that type III IFNs did not activate AKT or extracellular signal-regulated kinases [13] and a possible involvement of the alternative pathways in type III IFNs signaling is thereby controversial.

4. Growth Inhibitory Action

IFNs have a growth inhibitory action, which can represent one of the antiviral actions in host defense mechanism due to eliminating virally infected cells. Type I IFNs have been well documented to suppress growth of tumor cells through inducing apoptosis and cell cycle arrest. Procaspases are cleaved by IFN-α and IFN-β to induce apoptotic cell death, and Takaoka et al. showed that the type I IFNs augmented expression levels of the *p53* tumor suppressor gene, which suggest a close linkage between the antiviral function and the antitumor activity [21]. The p53 induction level by type I IFNs was however relatively low, and we believe that the induced p53 level will not activate apoptotic pathways [22]. Moreover, p53-mutated tumor cells were subjected to IFN-mediated apoptosis [23]; thereby, type-I-IFNs-mediated apoptosis can be rather p53 independent. Type III IFNs also induced apoptosis but it was observed in only some of cell lines derived from the same lineage. For example, esophageal carcinoma cells express the type III IFN receptors, and treatments with type III IFNs upregulated the MHC class I expression and produced antiviral molecules, 2′,5′OAS and MxA, in all the cells. Growth suppression by type III IFNs was thus observed in a third of esophageal carcinoma cells tested [24], suggesting the discrete pathways between the antiproliferative action and the other activities. A repertoire of type-I-IFNs-sensitive cells is the same as that of type III IFNs as far as we tested with the 9 kinds of esophageal carcinoma cells: type-I-IFNs-sensitive cells were also susceptible to type III and vice versa. It is interesting to know whether type III IFNs produce better growth inhibitory actions than type I IFNs. Maher et al. showed that type III IFNs produced greater growth inhibitory effects than IFN-α in a human keratinocyte cell line [19]. Direct comparison of the inhibitory ability between type I and type III IFNs is however difficult because the biological action per the IFN protein amount cannot be fairly judged. These data suggest that signal transductions involved in the growth inhibition are distinct from those of other functions such as antiviral activities but both type I and type III IFNs shared the same pathways pertinent to the growth inhibition.

The antiproliferative activity of type III IFNs was demonstrated in a certain type of tumors [25–27] and in nontumorous intestinal epithelial cells [17]. The scope of type III IFNs sensitivity is primarily dependent on the receptor expression as well as cell-type specificity as mentioned. The antiproliferative action, when more potent to tumors than to the normal counterparts, can be beneficial for cancer treatments. It is however relatively difficult to compare such preferential sensitivity with paired cell lines, normal and tumorous cells of the same cell origin. In esophagus, Het-1A, a nontumorous cell line immortalized with SV40 T antigen, is completely resistant to the type III IFNs-mediated growth inhibition despite being positive for the receptors. Some esophageal carcinoma cells however were also insensitive, and the preferential tumor susceptibility remains unknown in esophageal carcinoma. In addition, both intestinal epithelial cells and colon carcinoma cells were susceptible to type III IFNs [17]. Although no comparative data between tumors and nontumorous cells were available, the preferential inhibitory action to tumors may not be well evidenced. On the other hand, type I IFNs may have such propensity to induce cell death in tumors rather than nontumorous cells. The preferential inhibition could be linked with better proliferative activity of tumors compared with the normal counterparts but no conclusive data are currently available as to the preferential cytotoxicity to tumors with type I and type III IFNs.

The growth inhibition with type I and type III IFNs was directly evidenced by the decreased cell numbers as well as colorimetric assays. The activity is linked with tyrosine phosphorylation of IL-28Rα at residues of 343 and 517, which leads to optimal activation of STAT2 [28]. We recently demonstrated 2 modes of the growth inhibition, cell cycle arrest at G1-phase and apoptosis [24]. The cell cycle stop was accompanied by augmented p21 expression and pRb

dephosphorylation, which seem to be independent on p53 signaling pathways. The same biochemical changes were also demonstrated with murine tumor cells [27]. IFN-λ1 induced cell death in some of esophageal carcinoma cells by activating sequential caspase cleavage cascades including both intrinsic and extrinsic apoptotic pathways. We initially thought that IFN-λ1 induced G1 arrest and subsequently apoptosis but this was not the case. The choice of either G1 arrest or sub-G1 induction was dependent on the cell types. As mentioned, induction of G1-phase arrest or increased sub-G1 fractions by apoptosis was not observed in all the carcinoma cells tested. It is also interesting whether G1-arrested cells with type III IFNs were subjected to the same G1 arrest with type I IFNs. We found that type I IFNs did not induced such G1 arrest in the cells, suggesting a possible discrete pathways between type I and type-III-IFNs-mediated signaling. These studies suggested that the same cell repertoire within the identical lineage was susceptible to both type I and type III IFNs in the growth inhibitory action but the mechanisms were dependent on the cell type specificity.

5. Effects on Immune Systems

Type I IFNs have a wide range of immune stimulatory activities, but the main action is to augment T helper type 1 (Th1) cell responses, enhancing expression of MHC class I molecules and generating natural-killer- (NK-) cell- and T-cell-mediated cytotoxicity. Type I IFNs thus function to elevate both innate and adaptive immune responses. Type III IFNs seem to support cell-mediated immunity by upregulating the class I expression, but there has not been enough evidence to demonstrate that type III IFNs activate directly immune cells and induce production of Th1 cytokines. A recent study showed that IFN-λ1 diminished IL-13 levels and elevated IFN-γ production; however, subsequent study suggested that peripheral blood cells treated with IFN-λ1 rather upregulated expression levels of IL-6, IL-8, and IL-10 but not TNF-α or IL-1β, suggesting the role in Th2 differentiation but not in inflammatory reactions [29]. IFN-λ1 however has not been demonstrated to increase antibody formation despite augmented Th2 cytokine production. In addition, IFN-λ1 also elevated transcription of the *monokine induced by IFN-γ*(*Mig*) and the *IFN-γ inducible protein-10* (*IP-10*) genes and in peripheral blood cells [30]. These molecules favor for antiangiogenesis, which consequently suppress tumor growth. These data collectively imply that type III IFNs have similar immune regulatory activities as type I IFNs but could have some distinct properties. Moreover, type III IFNs activate STAT4 molecules which are not stimulated by type I IFNs through their phosphorylation, implying that type-III-IFN-mediated effects to immune systems are not identical to those with type I IFNs. Interestingly, Mennechet and Uzé reported contradictory data that type-III-IFNs-treated dendritic cells induced FOXP3-positive regulatory T cells [14] although meticulous further studies are required regarding the immune tolerance or the suppressive factions.

In *in vivo* settings, secretion of type III IFNs from tumors achieved antitumor responses against the transduced tumors. Numasaki et al. showed that local secretion of mIFN-λ2 from murine tumors produced antitumors responses which were mediated by neutrophils, NK and CD8-positive T cells [31]. The study also showed that IFN-γ but not IL-12, IL-17, or IL-23 was essential for the antitumor responses. Sato et al. demonstrated that NK and perhaps NKT cells played a crucial role in the antitumor effects with less significant involvement of cytotoxic T cells [27] although an *in vitro* assay showed that type III IFNs did not augment NK activities [13]. These results were inconsistent but suggest that type III IFNs induce immune responses, initially innate and sequentially adaptive immunity against tumors. In contrast, Lasfar et al. demonstrated intriguing results with murine B16 melanoma expressing mIFN-λ2 [7]. The growth of the transduced tumors was retarded, and even loss of the tumorigenicity was observed; however, mice that rejected the B16 tumors secreting mIFN-λ2 failed to induce immunological memory responses, suggesting that mIFN-λ2 does not contribute to adaptive immune responses. Moreover, they did not notice enhanced NK activities. These data suggest a possible mechanism by upregulated Mig and IP-10, both of which suppress neoangiogenesis within tumors. The mechanisms of cytotoxicity operating *in vivo* are different from that in *in vitro* studies, and several reasons besides the direct growth inhibitory action can explain the antitumor effects by type III IFNs, augmentation of MHC class I antigens expression which subsequently enhances antigenicity of tumors, a possible induction of Th1 type cytokines which increases antigen presenting and favors generation of cytotoxic T cells, and antiangiogenesis. These actions are also shared with type I IFNs and thereby specific immunological significance of type III IFNs remains unknown.

6. Conclusions and Prospects

Type III IFNs have multiple functions including antiviral, immunomodulatory, and antiproliferative actions, and the majority of the actions overlap with those of type I IFNs. A restricted expression of the type III receptor complex in contrast with an ubiquitous expression of type I IFNs receptors however suggests differential functions of the type III IFNs in *in vivo* settings. Several studies in fact demonstrated that differential activities between two types of IFNs in certain experimental models such as responsiveness to viral infections. Antitumor effects produced by type III IFNs may however not be different from those by type I IFNs except the tissues-dependent receptor distributions.

Feasible clinical applications of type III IFNs are determined by a number of factors including the biological activity and the potency. The antitumor effects *in vivo* of type III IFNs in comparison with type I IFNs are not well established, but the activities of type III IFNs seem to be less potent than those of type I IFNs from the standpoint of the MHC class I upregulation and the antiproliferative action although contradictory results were reported [19]. The restricted expression of type III IFNs receptors however can be a clue for the clinical application in term of cell-mediated delivery of type III IFNs to target tumors. Fibroblasts or endothelial cells, negative for type III IFNs receptors, are

resistant to the IFNs-mediated apoptosis but can deliver the IFNs to the target cells in the vicinity. Transduction of such carrier cells with the *IFN* genes and injection of the cells into type-III-IFNs-sensitive target tumors can generate antitumor effects by inducing apoptotic cell death. Cell-mediated delivery of a soluble factor can be more beneficial than systemic administrations since local concentrations of the factor are relatively maintained in the delivery system. Continuous secretion of factors from the producing cells can produce better therapeutic effects and circumvent any possible adverse effects.

Recombinant type I IFNs have been tested for the antitumor effects against a variety of tumors in clinical settings. The clinical studies however did not reveal any significant benefits partly due to the toxicity in systemic administrations. Any combinatory use with several types of anticancer agents did not increase the effects in most of the trials [32]. Type III IFNs have not yet been investigated for the clinical efficacy, but the similarity of its intracellular signal pathways with type I IFNs, despite several advantages of type III IFNs, implies that type III IFNs may not be dramatically better as an anticancer agent than type I IFNs. We probably need a novel strategy to obtain clinical benefits with type III as well as type I IFNs, which includes a local administration in the form of encapsulated protein particles and perhaps viral and nonviral expression vector systems. Much of preclinical studies and clinical trials with such a novel delivery system will be a subject in future.

Abbreviations

IFN: Interferon
STAT: Signal transducer and activator of transcription
IL: Interleukin-29
TLRs: Toll-like receptors
RIG-1: Retinoic acid inducible gene-I
JAK: Janus tyrosine kinase
TYK2: Tyrosine kinase 2
ISREs: IFN-stimulated response elements
ISGs: IFN-stimulated genes
IRF-9: IFN-regulatory factor-9
GAS: IFN-γ activation site
MxA: Myxovirus resistance protein
MHC: Major histocompatibility complex
PI3K: Phosphatidyl-inositol-3-kinase
MAP: Mitogen-activated protein
Th1: T helper type 1
NK: Natural killer
Mig: Monokine induced by IFN-γ
IP-10: IFN-γ inducible protein-10.

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Research Article

Tumor and Microenvironment Modification during Progression of Murine Orthotopic Bladder Cancer

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The aim of this study was to monitor changes in the expression of immune-related genes in the bladder after tumor implantation. Mice were orthotopically implanted with MB49-PSA cells (C57BL/6 mice) on day 1 and terminated on days 7, 14, 21, and 28. Another mouse model (MBT-2/C3H mice) was examined at day 7. Gene expression analysis was performed using a TaqMan Low Density Mouse Immune Panel (Applied Biosystems, USA) on RNA extracted from the bladders. Selected genes were reconfirmed by real-time PCR analysis and RT-PCR on the mRNA from other animals. Immune suppressive (IL13, IL1β, PTGS2, NOS2, IL10, CTLA4, and CCL22) and immune stimulatory genes (CSF2, GZMB, IFNγ, CXCL10, TNFα, CD80, IL12a, and IL6) and AGTR2 were increased by day 7. By day 28, IL10, CCL2, CCL5, CXCL11, CTLA4, GZMB, IFNγ, CSF2, and IL6 were significantly increased. Therapeutic strategies involving TH1 induction and TH2 dampening may improve responses to immunotherapy.

1. Introduction

Bladder cancer is the 7th most common cancer worldwide. Though bladder cancer is not usually life threatening, it is prone to recurrences which may progress to invasive cancer. Transurethral resection of the bladder tumor (TURBT) followed by Mycobacterium bovis, Bacillus Calmette Guerin (BCG) immunotherapy reduces the incidence of recurrence, but some 30–50% of patients do not respond to therapy [1, 2]. Recurrences are attributed to remnant tumor cells missed during surgery as a second surgical procedure prior to BCG therapy improves the response to therapy [1]. The immune response induced by BCG immunotherapy may inadvertently ensure the survival of less immunogenic remnant tumor cells which could give rise to recurrence and progression. The increased incidence of progression in patients who fail BCG immunotherapy gives some credence to this latter possibility [1].

Tumor immune editing is a dynamic process that has 2 important participants the tumor cells and the immune cells. Their interaction determines whether tumor regression or growth occurs. Orthotopic murine models of bladder cancer generated by implanting syngenic cell lines have been used to evaluate response to gene therapy. One such model, MB49 cells implanted in C57BL/6 mice, has been shown to be fairly similar to human bladder cancers [3]. Several cytokine genes have been evaluated for their ability to induce tumor regression in this model. Intravesical delivery of IL2 cured 40% of mice [4], IFNγ cured 50–80% of mice based on the amount of IFNγ secreting retrovirus delivered [5], TNFα cured 67% of mice [6], IFNα singly and/or with GMCSF cured 20–50% of mice [7, 8], adenovirus delivery of IL12 cured 88% of mice [9], and AdCD40 L delivery cured 60% of mice [10]. Tumor cells expressing IL12 and IL18 were completely rejected in mice [11]. The poor response to intravesical therapy has been attributed to the inadequacies of the gene delivery systems whether viral [12] or nonviral [13, 14]. However, MB49 cells were shown by Yang and Lattime [15] and Halak et al. [16] to induce the expression of IL10 in the bladder. IL10 is an immunosuppressive gene and its expression may have contributed to the poor response. Besides IL10, there may be other immunosuppressive mechanisms involved in the interplay between the immune system and the tumor cells.

The aim of this study therefore was to characterize the changes in the bladder environment after orthotopic tumor

cell implantation. In general, tumors implanted in the bladder if not treated result in death after about 4–6 weeks. Some mice may show spontaneous cures in this time period. To understand the events that occur during tumor growth in the bladder environment, we chose to evaluate gene expression changes soon after tumor cell implantation (7th day) and at a later stage (28th day). Several immunosuppressive genes were induced after tumor implantation, and these represent possible new targets for therapy. A few mice were also examined 14 and 21 days after tumor implantation. Our results indicate that the TH1/TH2 balance in the tumor environment varies with time and may influence the success of any therapy.

2. Methods

2.1. Tumor Implantation. All animal work adhered to the National University of Singapore, Institutional Animal Care and use Committee (IACUC), guidelines on animal use and handling. Four to six-week-old female C57BL/6 mice were orthotopically implanted with MB49-PSA (prostate-specific antigen secreting MB49 cells) [8] using poly-L-lysine (PLL) as described by Ninalga et al. [17]. Mice were anesthetized with 75 mg ketamine/kg and 1 mg medetomedine/kg of animal weight (0.1 mL/10 g mice body weight) prior to implantation. MB49 cells were originally obtained from Prof T Ratliff, Purdue University. Briefly, mice were given a bladder instillation of 0.1 mL of sterile 0.01% PLL (Sigma-Aldrich, St. Louis, Mo, USA) for 20 mins prior to instillation of the tumor cells (1×10^6 cells/mL) for 2 hours. Control mice were treated with PLL but not implanted with tumor cells. PSA levels in urine were monitored using a free-PSA chemiluminescence ELISA kit (Autobio Diagnostics, Zhengzhou, China) and normalized against creatinine levels using a kit (Wako Pure Chemicals Industries, Osaka, Japan). Mice were killed on days 7 and 28 and RNA isolated from the bladders. For gene expression studies, 10 mice were used per group. To determine gene expression changes with time, at least 3 mice were sacrificed on days 7, 14, 21, and 28. For flow cytometry studies, 6 mice were sacrificed on day 28 and tissues were pooled from 2 mice for analysis.

Similarly, four-to-six-week-old female C3H/HeJ mice were orthotopically implanted with MBT2-PSA (prostate-specific antigen secreting MBT2 cells) while control C3H/HeJ mice were treated with PLL only. After 7 days, 4 mice from each group were sacrificed for gene expression analysis.

2.2. RNA Isolation and Real-Time LDA Panel. Frozen bladders and lymph nodes were soaked overnight in RNA*later*-ICE (Ambion, Austin, Tex, USA) at −20°C before they were homogenized in TRIzol Reagent (Invitrogen, Carlsbad, Calif, USA). CDNA was reverse transcribed from total RNA (10 μg per sample) with random primers using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, Calif, USA). MRNA expression was analyzed using a TaqMan Low Density Mouse Immune Panel (Applied Biosystems) containing a panel of 96 immune-related genes (cytokines, chemokines, growth factors, immune regulators, apoptosis markers, ischemia markers, tissue-specific markers, and others including classic and endogenous controls) as described by Cai et al. [18]. Detection and analysis was performed on the ABI PRISM 7900HT Sequence Detection System with ABI 7900HT SDS Software Version 2.2.1 with the following parameters: 2 mins at 50°C, 10 mins at 94.5°C, and 40 cycles of 30 s at 97°C for denaturation and 1 min at 59.7°C for transcription. Differential gene expression profiling was performed using the comparative C_T method of relative quantitation. All samples were loaded in triplicate, and results with C_T SD $\geq$ 0.3 were discarded. The lowest limit of detection is C_T = 32 thus any gene with a C_T value beyond 32 is considered not detectable. Only genes displaying at least a 2-fold difference in expression level relative to control were considered to be upregulated. Gene expression was confirmed using real-time PCR analysis for a select group of genes.

2.3. Real-Time PCR Analysis and RT-PCR. Real-time PCR reactions for single genes were performed using 100 ng of reverse transcribed RNA, TaqMan universal PCR master mix and pre formulated 20x TaqMan gene expression assay in a 96-well PCR plate. The genes and their TaqMan gene expression assay numbers are Actb Mm00607939_s1; AGTR2 Mm00431727_g1; CSF2 Mm00438328_m1; CTLA4 Mm00486849_m1; CXCR3 Mm00438259_m1; GAPDH Mm99999915_g1; GZMB Mm00442834_m1; IFNγ Mm00801778_m1; IL10 Mm00439616_m1; PTGS2 Mm00478374_m1; TGFb1 Mm00441724_m1; TNFα Mm00443258_m1. Detection and analysis was performed on the ABI 7500 Real-Time PCR System with the ABI 7500 System Sequence Detection Software Version 1.3.1. PCR was performed for forty cycles with the following parameters: 2 mins at 50°C, 10 mins at 95°C, and for each cycle 15 s at 95°C for denaturation and 1 min at 60°C for transcription. All samples were measured in triplicates and normalized with beta actin. The lowest limit of detection is C_T = 35.

Semiquantitative PCR was performed as described before [19] for 35 cycles. The PCR products were separated on agarose gels, and band intensities were quantified with Gene Tools analysis software (SynGene, Cambridge, England) and normalized against GAPDH or beta actin and expressed as relative quantitation (RQ). Table 1 lists the genes, primer sequences, annealing temperature, and the size of the PCR products.

2.4. Flow Cytometry Analysis. Bladders were harvested, placed in RPMI-1640, and cut into smaller pieces before digestion with collagenase (Sigma-Aldrich) for 30 mins at 37°C. The suspension was filtered through a 70 μm cell strainer (BD, Franklin Lakes, NJ, USA) and centrifuged at 10.4 g for 5 mins at 4°C. The samples were incubated in RBC lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) for 5 mins at room temperature and rinsed twice in cold PBS. The number of cells in each sample was enumerated with a haemocytometer. Pooled samples were resuspended in buffer containing 1% (w/v) BSA and 0.01% sodium azide in 1xPBS. Samples were assessed for T cells

Table 1: Primer sequences, annealing temperature, and fragment length of PCR products.

Gene	Primer sequence (5′–3′)	Temp. (°C)	Size (bp)
ACTB F	ACATGGAGAAGATCTGGCAC	58	660
ACTB R	CAGACAGCACTGTGTTGGCA		
CCL2 F	GCATCCACGTGTTGGCTCAG	60	383
CCL2 R	CACACTGGTCACTCCTACAG		
CCL22 F	CGTCCTTCTTGCTGTGGCAA	60	233
CCL22 R	CTTCTTCACCCAGACCTGCC		
CCL3 F	GCAACCAAGTCTTCTCAGCG	58	194
CCL3 R	CTTGGACCCAGGTCTCTTTG		
CCL5 F	GGTACCATGAAGATCTCTGC	51.5	286
CCL5 R	CTATCCTAGCTCATCTCC		
CCR2 F	GAGCCTGATCCTGCCTCTAC	58	371
CCR2 R	GGCACTGTTTGAAGAGACGT		
CD80 F	GCAGGATACACCACT	55	480
CD80 R	GGAAGCAAAGCAGG		
CSF2 F	TGGCCTGGGCTTCCTCAT	60	311
CSF2 R	GGATGACATGCCTGTCAC		
CXCL10 F	CGTGGTCACATCAGCTGCTA	58	244
CXCL10 R	TAGAACTGACGAGCCTGAGC		
CXCL11 F	AGGTCACAGCCATAGCCCTG	62	251
CXCL11 R	CCTGCATTATGAGGCGAGCTTGC		
FOXP3 F	TCGTAGCCACCAGTACTCAG	57	386
FOXP3 R	ATCTACGGTCCACACTGCTC		
GAPDH F	CACCCTGTTGCTGTAG	60	900
GAPDH R	GTCGGTGTGAACGGAT		
GATA3 F	GATAGCATGAAGCTGGAGACG	60	500
GATA3 R	AAGCTTGTAGTACAGCCCACA		
IFNg F	ACTGCCACGGCACAGTC	60	389
IFNg R	CCGCTTCCTGAGGCTG		
IL12a F	CCATCGATGAGCTGATGCAG	58	340
IL12a R	ATGCTGAGGTAGCTGTGCCA		
IL13 F	TGTCTCTCCCTCTGACCC	60	201
IL13 R	TACAGAGGCCATGCAATATCC		
IL15 F	ATGAACTGCTTTCTCCTGGAA	60	205
IL15 R	TGGACAATGCGTATAAAGCTTTGC		
IL17 F	CCAGGGAGAGCTTCATCTGT	58	431
IL17 R	AAGATGCTGGTGGGTGTGGG		
IL1b F	GGCAACTGTTCCTGAACTCAACTG	62	739
IL1b R	GCTTGTCTGCTGCTTGTGAGGTGC		
IL2 F	TGATGGACCTACAGGAGCTCCTGAG	60	191
IL2 R	GAGTCAAATCCAGAACATGCCGCAG		
IL6 F	GATGCAACCAAACTGGATATAATC	60	225
IL6 R	GAGCATTGGAAGTTGGGGTA		
NOS2 F	AAAGCCACGAGGCTCTGACA	58	259
NOS2 R	ACCATCACGCTCGAGGTTGA		
PTGS2 F	ATGCTCTTCCGAGCTGTGCT	55	239
PTGS2 R	GTGGGTCAGGATGTAGTGCA		
TBET F	CTAAAGCTCACCAACAACAAG	55	840
TBET R	GTTGGGAAAATAATTATAAAA		

Table 1: Continued.

Gene	Primer sequence (5′–3′)	Temp. (°C)	Size (bp)
TGFb1 F	CTGCAAGACCATCGACAT	55	580
TGFb1 R	ACAAGAGCAGTGAGCGCT		
TNF F	TGCACCACCATCAAGGACTC	58	360
TNF R	CAGCTCAGCTCCGTTTTCAC		

using fluorescein isothiocyanate- (FITC-) conjugated anti-CD3, phycoerythrin- (PE-) conjugated anti-CD4 and anti-CD8a. Macrophages and NK cells were detected with anti-Mac3-FITC and anti-Pan NK-PE. All antibodies were from BD. Flow cytometry was performed on a $CyAn_{ADP}$ (Dako Cytomation, Sweden). The data obtained was analyzed with the Summit software.

2.5. Statistical Analysis. The SPSS statistical package (SPSS, Ill, USA) was used to analyze statistical significance. The *t*-test for equality of means was used to analyze the gene expressions of two groups of animals (normal and tumor bearing). To analyze variance between the gene expressions in the different groups of animals, one-way ANOVA (Bonferroni) was used. Bivariate correlation was used to analyze correlation between two arrays. A P value of ≤ 0.05 was deemed to be significant.

3. Results

3.1. Tumor Growth and Gene Expression. The murine bladder cancer cell line MB49 was modified to secrete PSA so that PSA levels could be used as a surrogate marker for the presence of tumors [8]. MB49-PSA, like the parental MB49, is fairly immunogenic, and some mice were spontaneously cured (35%) after tumor implantation. MB49 cells express the HY antigen as they were originally isolated from a male mouse [20] as well as BLCA-4, PSCA, and STEAP [21] and consequently are immunogenic, and some mice may become cured without any therapy [6, 7]. The tumor implantation technique used has a 100% implantation efficiency [17], and this was confirmed by measuring PSA secretion in the urine. The growth of the tumors in the bladder was also monitored by measuring urinary PSA levels. Figure 1(a), top right panel shows tumor bearing bladders harvested at day 7, when the tumors were small and at day 28 (bottom right panel) when the tumors were large. In all mice, an initial increase in urinary PSA secretion was observed at day 11 but later as some mice were cured, PSA secretion decreased, Figure 1(b).

To confirm the presence of tumors at the point of termination of the study, PSA mRNA levels were determined by real-time PCR analysis, Figure 1(c). This is especially important in mice with low PSA protein levels in urine as real-time PCR analysis is a more sensitive method to confirm the presence of tumors. RNA isolated from samples with confirmed tumors was used to probe a real-time gene expression array of cytokine/chemokine genes. For each time point, two independent samples were analyzed on the arrays. The correlation between the arrays is shown in Figures 1(d) and 1(e). The data from the day 7 arrays showed greater homology than those from the day 28 arrays indicating the greater diversity of the evolving tumor and the tumor microenvironment. To validate the array data, 9 genes were selected to be reconfirmed by real-time PCR using the same mice samples that were used on the array. The LDA data was comparable with the data obtained from real-time analysis, Figure 1(f) confirming the validity of the array data.

The genes up- and downregulated on both arrays are summarized in Figure 1(g). Seventeen genes were upregulated in both day 7 and day 28 bladders as shown in Figure 1(g). At Day 7, proteins associated with MHC class I and II receptors (β2M, CD3e, H2-EB1, HMOX1, and PTPRC) and monocytes and dendritic cells (CD40, CD80, and CD86) chemokine and cytokine genes (CCL2, CCL5, CSF3, CXCL10, CXCL11, GZMB, IFNγ, IL1b, NOS2, PGK1, PRF1, SOCS1, and TNF) and receptors (IL2ra) attachment and trafficking proteins (CCR2, VCAM1) and the transcription factors that regulate their expression (STAT1, NFKB2) were upregulated. Genes upregulated uniquely by day 28 include CCL3, CCR7, CD8a, CSF2, FASL, IL10, IL1a, IL6, PTGS2, and STAT4. IL10, PTGS2, and FASL are known to suppress the immune response and SMAD7 which inhibit TGFβ, and bone morphogenetic protein expression was downregulated which would lead to an environment favoring tumor growth. But there were increased CSF2, CCL3, and IL6 which would recruit immune cells as well as increased CD8a expression. Thus, the tumor environment is a dynamic one with infiltrating immune cells trying to destroy the tumor in an environment that can be inhibitory to their activation and/or action.

3.2. Validating Gene Expression Changes and Immune Cell Infiltration in Tumor Bearing Mice. To validate the array data, the expression of selected genes was assayed in other mice. This included 17 of the differentially expressed genes from Figure 1(g) and 12 that were determined to be similar to controls. The expression of these genes in MB49 cells was also examined. Table 2 summarizes the results of the analyses which were performed using either real-time analysis or semiquantitative PCR.

Several of the genes, whose expression was increased in the bladder at day 7, were found to be expressed by MB49 cells, such as CSF2, PTGS2, CXCL10, TGFβ1, CCL2, and CCL5. The increased expression of IFNγ in the bladder at day 7 is probably due to CXCL10 being secreted by the tumor cells (Table 2), but MB49 itself does not express IFNγ.

Increased expression of CD80, GZMB, and CTLA4 is probably due to immune cell infiltration of the bladder at

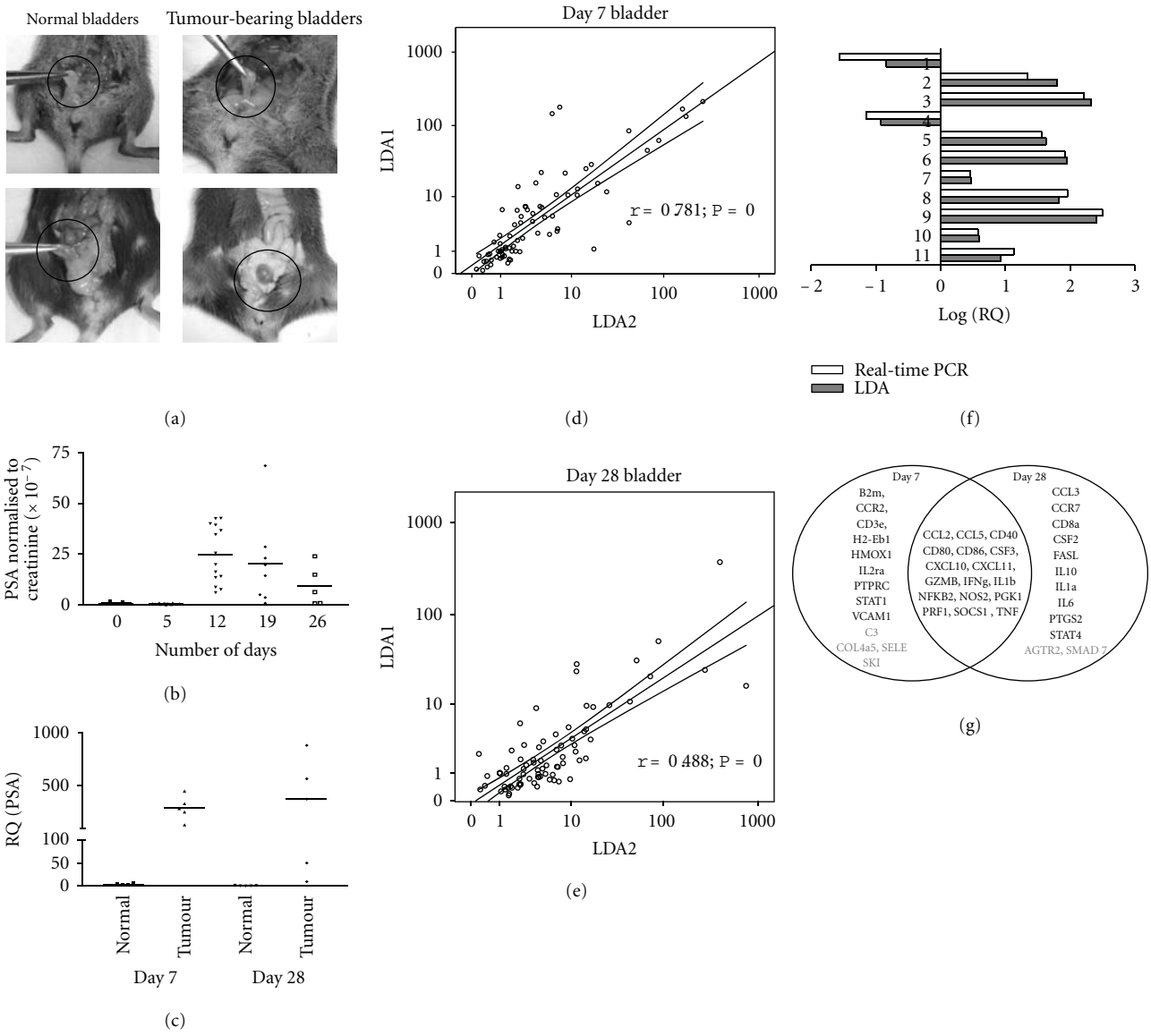

Figure 1: An orthotopic murine model of bladder cancer was established and gene expression analysis performed on day 7 and day 28. (a) Neovascularization was apparent in the bladders 7 days after tumor implantation (upper panel), and after 28 days, the tumor-bearing bladder appeared highly vascular and larger than normal bladders (lower panel). (b) Tumor growth was monitored by measuring urinary PSA which was normalized with creatinine. (c) To confirm the presence of tumors, PSA gene expression in the bladder was analyzed by real-time PCR when the mice were culled at day 7 and day 28. Gene expression in the bladder was analyzed using the LDA arrays and 2 individual mice bladders. The scatter diagrams show the relationship between the RQ values of the 2 arrays of the 2 mice for the (d) day 7 and (e) day 28 bladders. The axes of the scatter diagrams are in the logarithmic scale. The middle line is the best-fit line and the two lines flanking represent the 95% confidence interval. r is the Pearson's correlation, and p is the significance level. To reconfirm the array data several genes were reanalyzed by real-time PCR using the same murine samples. (f) The log (RQ) shows the same profile for all the genes evaluated. The 11 genes are (1) AGTR2; (2) CTLA4; (3) IFNγ; analyzed on RNA from mouse sample one and (4) AGTR2; (5) CSF2; (6) CTLA4; (7) CXCR3; (8) GZMB; (9) IFNγ; (10) PTGS2 and (11) TNFα analyzed on RNA from mouse sample 2. (g) A summary of genes with increased or decreased expression on days 7 and 28 after tumor implantation.

day 7. By day 28, GZMB and CTLA4 were still significantly increased and CD80 was elevated compared to control animals. Immune cells in the bladder at day 28 were analyzed using flow cytometry, Table 3. There was increased $CD3^+CD8^+$ in the bladder at day 28 which was consistent with the increased CD8a expression noted on the array. B cells were significantly increased in the bladder at day 28, Table 3. The presence of CTLA4 may explain the lack of tumor reduction despite infiltrating immune cells. CTLA4 binds to CD80 on antigen-presenting cells and blocks the activation of T cells. The transcription factors TBET, GATA3, and FOXP3 regulate TH1, TH2, and Treg development.

Table 2: Relative expression of genes in C57BL/6 mice bearing bladder tumors.

		Day 7		Day 28		
	Gene	Control (7)	Tumor (12)	Control (11)	Cured (4)	Tumor (15)
Realtime†	AGTR2	0.871 ± 0.123	0.231 ± 0.250*	1.381 ± 0.623	1.072 ± 0.251	0.171 ± 0.205*
	CSF2	0.944 ± 0.218	26.07 ± 28.00*	2.621 ± 5.554	1.637 ± 0.443	38.10 ± 37.28*
	CTLA4	1.088 ± 0.567	38.27 ± 36.06*	2.421 ± 6.216	3.026 ± 0.834	47.19 ± 30.92*
	GZMB	0.927 ± 0.312	27.07 ± 25.39*	0.966 ± 0.291	2.495 ± 0.456	7.349 ± 7.158*
	IFNg	1.178 ± 0.455	127.5 ± 127.9*	0.726 ± 0.542	2.005 ± 0.742	8.875 ± 10.89*
	IL10	5.022 ± 8.895	17.94 ± 36.01	1.377 ± 0.981	2.526 ± 0.975	3.749 ± 2.838*
	PTGS2	1.126 ± 0.781	2.424 ± 0.789*	1.245 ± 0.844	3.818 ± 3.132	3.040 ± 6.290
	TGFb1	0.934 ± 0.302	2.450 ± 2.500	1.251 ± 0.576	3.885 ± 3.301	2.110 ± 1.439
	TNF	0.956 ± 0.155	11.36 ± 12.66*	2.658 ± 5.417	3.333 ± 2.303	8.008 ± 14.67
	PSA	2.157 ± 1.898	126.7 ± 163.7*	0.491 ± 0.532	0.095 ± 0.099	243.4 ± 258.1*
RT-PCR	CCL2	1.140 ± 0.510	2.200 ± 0.840	0.799 ± 0.398	1.350 ± 0.616	1.996 ± 0.630*
	CCL22	0.063 ± 0.026	0.212 ± 0.062*	0.095 ± 0.019	0.139 ± 0.050	0.108 ± 0.070
	CCL3	0.193 ± 0.340	0.073 ± 0.044	0.928 ± 0.397	1.699 ± 2.210	2.366 ± 1.533
	CCL5	0.308 ± 0.206	0.613 ± 0.284	0.360 ± 0.050	0.670 ± 0.170	0.680 ± 0.090*
	CD80	0.094 ± 0.055	0.367 ± 0.094*	0.016 ± 0.008	0.134 ± 0.268	0.631 ± 0.825
	CXCL10	2.383 ± 1.339	591.9 ± 257.8*	1.854 ± 1.257	4.831 ± 2.943	17.04 ± 30.28
	CXCL11	0.523 ± 0.351	1.445 ± 0.374	0.297 ± 0.390	0.854 ± 0.656	2.240 ± 2.576*
	FOXP3	0.016 ± 0.007	0.217 ± 0.203	0.042 ± 0.018	0.068 ± 0.058	0.155 ± 0.141
	GATA3	0.901 ± 0.327	0.118 ± 0.039*	0.858 ± 0.173	0.935 ± 0.203	0.584 ± 0.244
	IL12a	0.014 ± 0.008	0.064 ± 0.031*	—	—	—
	IL13	0.020 ± 0.010	0.600 ± 0.220*	0.118 ± 0.277	0.060 ± 0.063	0.033 ± 0.013
	IL15	1.295 ± 0.685	0.773 ± 0.381	0.298 ± 0.149	0.218 ± 0.255	0.225 ± 0.189
	IL1b	0.125 ± 0.230	1.174 ± 0.578*	0.006 ± 0.003	0.011 ± 0.005	0.211 ± 0.177
	IL2	0.681 ± 0.714	0.994 ± 0.609	2.098 ± 1.635	1.764 ± 0.804	1.582 ± 1.329
	IL6	1.550 ± 0.670	3.190 ± 0.710*	0.829 ± 0.099	1.482 ± 1.026	4.957 ± 2.054*
	NOS2	0.580 ± 0.100	0.950 ± 0.230*	—	—	—
	TBET	0.057 ± 0.009	0.051 ± 0.013	0.083 ± 0.068	0.097 ± 0.068	0.075 ± 0.035

Note. The sample size for each group is indicated in brackets and "—" indicates not done. †Data is presented as the mean RQ ± SD. *indicates $P < 0.05$ with respect to control.

GATA3 was low in the bladder of tumor-bearing mice at day 7, and FOXP3 was increased indicating the presence of suppressor cells.

Using PSA expression as a measure of tumor size, we segregated the mice as cured and mice bearing small tumors (mean PSA expression 73.03 ± 69.92), medium tumors (mean PSA expression 311.2 ± 45.85), and large tumors (mean PSA expression 1602 ± 1914) for the day 28 samples, Table 4. There were a clear and significant decrease in AGTR2 expression and increase in CTLA4 and GMCSF expression with increasing PSA levels. Further, it appeared that the large tumors expressed high levels of TH1 cytokines like IFNγ and even TNFα. Thus, there must be immune-suppressive factors blocking tumor eradication.

As a further test of the robustness of the data obtained, another mouse tumor model, C3H mice bearing MBT-2 tumors, was also evaluated. About 36.8% (7/19) of the genes, namely, CSF2, TNFα, CCL22, CXCL10, IL13, IL1b, and IFNγ were significantly upregulated in both mice models by day 7.

Table 3: Immune cells present in the bladder at day 28.

Markers	Control	Tumor
$CD3^+CD4^+$	0.593 ± 0.577	1.387 ± 1.171
$CD3^+CD8a^+$	0.000 ± 0.000	2.460 ± 0.716*
$CD4^+CD25^+$	4.203 ± 4.076	1.810 ± 0.605
$CD45R/B220^+$	0.043 ± 0.075	2.037 ± 0.926*
pan-NK^+	7.003 ± 1.851	5.613 ± 2.470
$Mac3^+$	9.670 ± 1.769	14.54 ± 6.187

* denotes $P < 0.05$ with respect to control.

3.3. The Immune Environment Shapes the Tumor. To monitor the change in gene expression with time, bladders were also harvested at day 14 and 21 after tumor cell implantation and examined for the expression of IFNγ, CXCL10, TGFβ, IL10, and PTGS2. IL10 and IFNγ were chosen as these are not produced by the tumor cell line and their presence denotes expression by immune or other urothelial cells. Further,

Table 4: Bladder gene expression at day 28 segregated by PSA expression.

			Day 28		
Group	Control	Cured	Tumor (small)	Tumor (medium)	Tumor (large)
Gene/no	11	4	5	6	4
PSA	0.491 ± 0.532	0.095 ± 0.099	73.03 ± 69.92	311.2 ± 45.85	1602 ± 1914*
AGTR2	1.381 ± 0.623	1.072 ± 0.251	0.342 ± 0.249*	0.018 ± 0.011*	0.187 ± 0.130*
CSF2	2.621 ± 5.554	1.637 ± 0.443	18.16 ± 21.55	28.88 ± 12.69	76.86 ± 52.56*
CTLA4	2.421 ± 6.216	3.026 ± 0.834	16.42 ± 15.00	68.02 ± 26.53*	54.42 ± 21.72*
GZMB	0.966 ± 0.291	2.495 ± 0.456	8.394 ± 11.47*	5.305 ± 2.977	9.108 ± 5.886*
IFNg	0.726 ± 0.542	2.005 ± 0.742	10.54 ± 15.36	4.594 ± 3.200	13.22 ± 12.45*
IL10	1.377 ± 0.981	2.526 ± 0.975	5.493 ± 4.593*	3.301 ± 1.454	2.677 ± 2.072
PTGS2	1.245 ± 0.844	3.818 ± 3.132	1.877 ± 2.350	4.594 ± 10.00	2.163 ± 1.499
TGFb1	1.251 ± 0.576	3.885 ± 3.301	1.669 ± 1.321	1.826 ± 0.698	2.835 ± 2.072
TNF	2.658 ± 5.417	3.333 ± 2.303	3.148 ± 3.013	3.721 ± 1.815	20.51 ± 26.49*

Data is expressed as the mean RQ ± SD. *N*: number of mice samples analyzed; "—": not done. "*" Significant ($P < 0.05$) with respect to control.

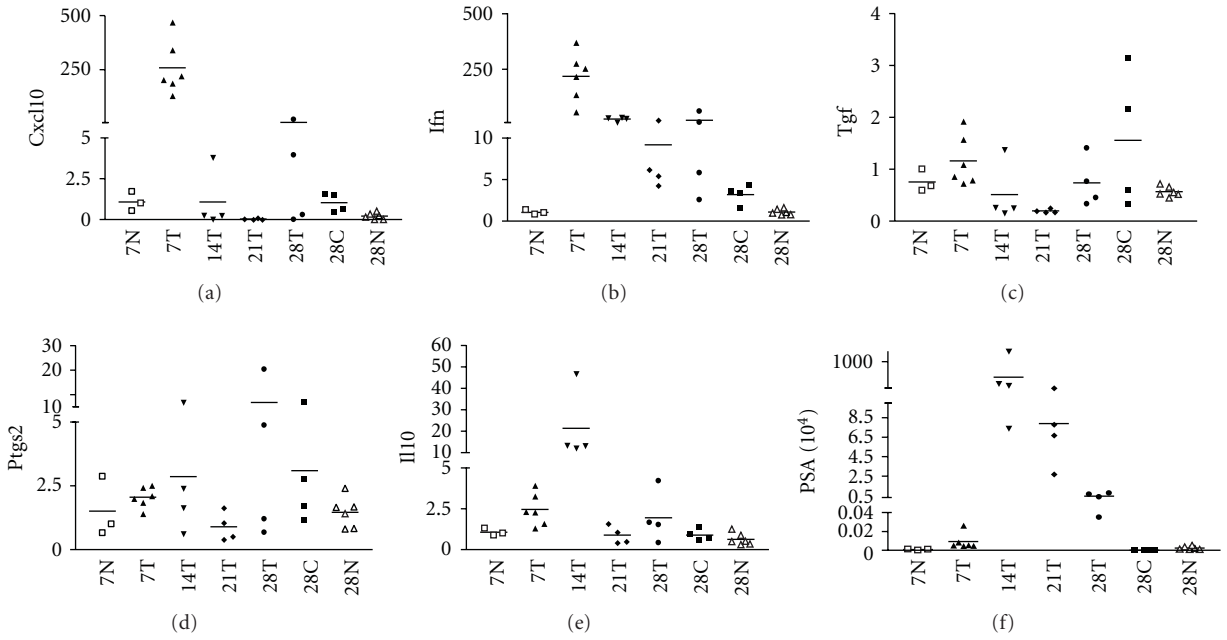

Figure 2: Monitoring gene expression changes in the bladder days after tumor implantation. The genes analyzed were (a) CXCL10; (b) IFNg; (c) TGFb; (d) PTGS2; (e) IL10; (f) PSA. Samples obtained from normal healthy bladders are labeled N, those from tumor bearing mice are labeled T, and samples obtained from cured mice are labeled C. Each point represents one murine bladder sample. * indicates a significant difference ($P < 0.05$) with respect to all the other groups.

both these cytokines were upregulated in the two tumor models that were evaluated. IFNγ decreased with time as did CXCL10, but not as dramatically as CXCL10. This was not a result of tumor reduction but of the process of selection for a less immunogenic cell type as PSA levels did not decrease as dramatically (Figures 2(a) to 2(d)). Analysis of day 14 and day 21 samples showed that the decrease in CXCL10 from days 7 to 14 was followed by a concomitant increase in IL10 over the same period. Thus, while the tumor changes the environment in the bladder, as shown by the increase in IFNγ and IL10, it too is changed by the activity of the immune cells recruited to the bladder which selectively destroy the immunogenic cells and thus encourage the survival of less immunogenic cells.

4. Discussion

A comparison of the gene expression patterns in tumor-bearing animals revealed the variability of gene expression, as there were only a 55% homology at day 7 and a 41.6% homology at day 28 in gene expression between mice. This indicates the heterogeneous nature of the tumor cells that

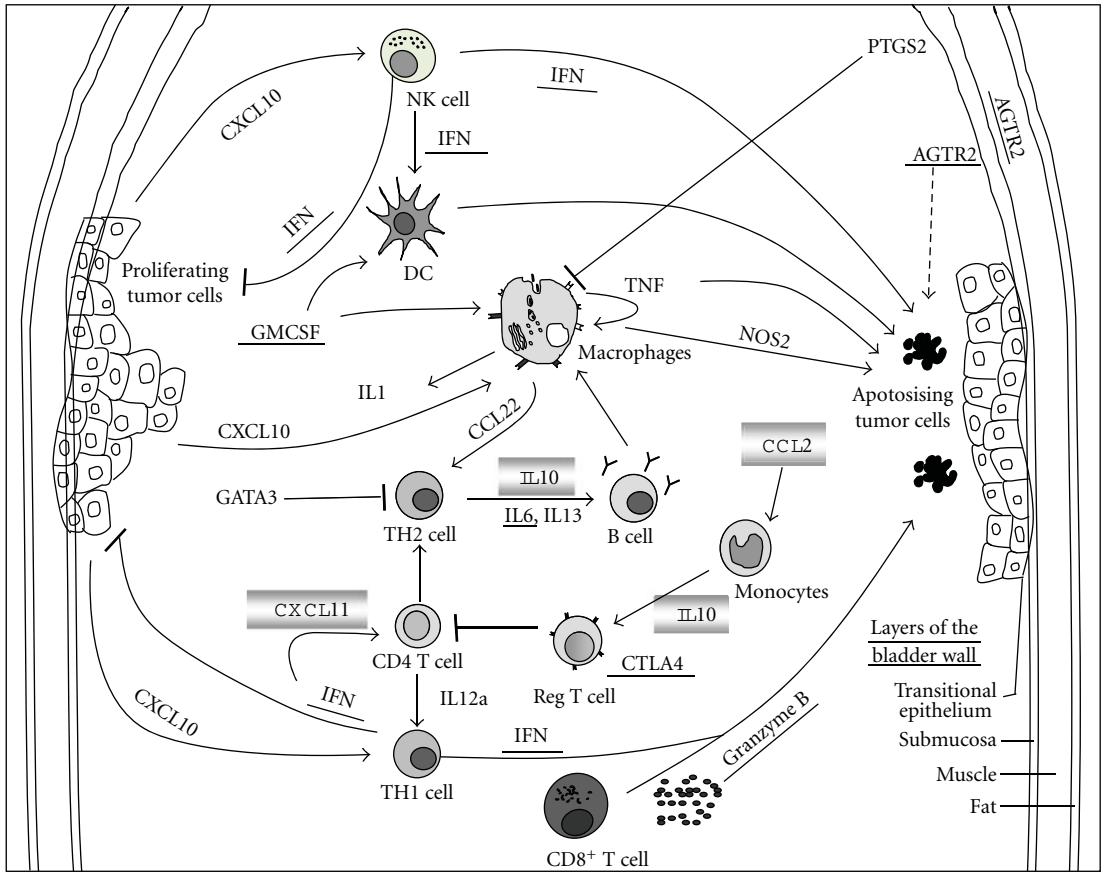

Figure 3: A schematic diagram that summarizes the gene expression changes and immune cells identified in bladders after tumor implantation. Proliferating tumor cells increase the expression of GMCSF and CXCL10 which in turn recruit immune cells such as natural killer (NK) cells, dendritic cells (DC), macrophages, and T cells to the bladder. Increased expression and production of IFNγ, TNFα, IL12a, NOS2, and GZMB by the immune cells would inhibit tumor proliferation and induce apoptosis. Transcription factor GATA3 was downregulated, suppressing the production of TH2 T cells. CTLA4 binds to CD80 and inhibits T-cell activation. Upregulation of PTGS2 in either tumor or immune cells or both would have an inhibitory effect on immune cells. Downregulation of AGTR2 could have occurred in the tumor cells, normal bladder epithelia, or submucosa. The dotted arrow indicates that AGTR2 may cause apoptosis in tumor cells. Genes that are underlined were present at both day 7 and day 28. Genes in the shaded box were found only at day 28. Genes whose expression was upregulated are indicated by arrows pointing up, and those that were downregulated are indicated by arrows pointing down.

were implanted. As tumors grew in the bladder, there were both sculpting of the tumor cells by immune cells as well as suppression of the immune cells infiltrating the tumor by the tumors themselves.

By day 7, IFNγ, CSF2, CXCL10, GZMB, PTGS2, TNFα, CD80, CCL22, IL12a, IL13, IL1b, IL6, NOS2, and CTLA4 were significantly upregulated in the tumor-bearing mice bladders and AGTR2 and GATA3 were downregulated. These genes could lead to active immune cell recruitment (CSF2, CXCl10, and IFNγ) and modulation of immune cell activation (IL12a, IFNγ, CSF2, CD80, GZMB, NOS2, and CTLA4), and the generation of an inflammatory environment (IL6 and IL1b). Based on the genes identified granulocytes, NK cells and T cells would be attracted by CSF2, CXCL10, and CCL22 to the bladder. As a consequence of the presence of NK and T cells, there would be increased expression of GZMB and IFNγ Figure 3. TNFα could induce direct cytotoxic effects on tumor cells [6]. The inhibitory activity of IL13, IL1b, PTGS2, NOS2, and CTLA4 [22–26] on the immune response would enhance tumor growth. CCL22 has been linked to the recruitment of suppressor T cells [27], but it also recruits other immune cells which may reduce tumor growth in the absence of Tregs. Similarly, IL13 has been reported to be both pro- and antitumor [22, 28]. By day 28, IL10, CCL2, CCL5, and CXCL11 which were slightly increased at day 7 were significantly increased in the bladder. Also CTLA4, GZMB, IFNγ, TNFα, and IL6 remained significantly increased in the bladder, Figure 3. When gene expression was analyzed with respect to tumor sizes, both immunosuppressive (CTLA4) and immune stimulatory (IFNγ and TNFα) genes were increased with increasing tumor size.

Increased CD8 T cells and B cells were found in the bladder at day 28. In human urothelial cancer tissue, increased tumor infiltrating CD8 T cells correlates with better disease-free survival [29]. However, we have previously found that immune cells recruited to the bladder are usually located at the periphery of the tumor mass [30] and may thus

be ineffective in the tumor environment. This could be caused by the immunosuppressive genes identified in this study such as CTLA4. The MB49 cells express several tumor antigens as do human bladder tumors [31]. It is likely that immunosuppressive molecules induced by the tumors may prevent the activation of an adequate immune response against the tumor antigens. In support of this view, bladder cancer patients receiving anti-CTLA4 therapy showed an increase in the ratio of effector to regulatory T cells as well as tumor antigen-specific CD4 T cells [24].

AGTR2 was consistently downregulated in all tumor bearing samples. This receptor downregulates the stimulatory effect of EGF on the growth of prostate cancer cells [32]. AGTR2 acts with TIMP3 to block angiogenesis [33], but in AGTR2 knockout mice there was impaired induction of peripheral angiogenesis [34]. Identifying the cells expressing AGTR2 may clarify the likely role of AGTR2 in angiogenesis. AGTR2 expression has been associated with apoptosis in prostate cancer cells [35]. It needs to be determined if AGTR2 has a similar function in human bladder cancer cells. If it does have a similar effect, then reintroduction of this gene may eradicate tumors in the bladder.

Genes that were upregulated at day 7 represent early changes that have occurred in the bladder, while those that appear later may represent genes that promote survival of the tumor. Both MB49 and MBT-2 cells express the chemokine CXCL10 (IP10) as do human bladder tumors [36] and this chemokine has proinflammatory and angiostatic effects in the tumor microenvironment [37]. FOXP3-positive T cells and increased IL10 levels are present in human bladder cancers [38] and in the mice models. IL1b, IL6, GMCSF, MCSF, GCSF, and TNFα are expressed in a human bladder carcinoma cell line [39]. Local production of CCL2 (monocyte chemoattractant protein-1 (MCP-1)), in patients, correlated significantly with bladder cancer progression [40]. CCL22 (macrophage-derived chemokine (MDC)) and NOS2 have been shown to be expressed in clinical tissue specimens [36, 41]. Blocking CCL22 with siRNA during the differentiation and maturation of DC can block Treg recruitment [42], thus this is a good target for inhibition of tumor-induced immune suppression. IL13 suppression has both pro- and antitumor effects depending on the cell type producing it [22, 43]. A role for IL13 in human bladder cancer has not been determined as yet. PTGS2 or cyclooxygenase 2 (COX2) was found to be positive in 60% (368/617) of bladder cancer tissue [44]. In human urothelial cancer, TGF beta has been shown to correlate with disease progression [45]. Thus, the murine models are representative of the human disease and the new genes identified as being differentially expressed in this study may represent new targets for therapy of human bladder cancers.

Our data shows that there are both TH1 and TH2 genes upregulated by the presence of tumor cells. Arum et al. who evaluated a rat orthotopic model of bladder cancer also found that host immune response pathways were actively upregulated after tumor implantation [46]. Any cytokine that tilts the TH1/TH2 balance in favor of a TH1 response may induce tumor regression. It was recently reported that tilting the TH1/TH2 balance may block the immune suppression of CD8 T cells by mononuclear phagocytes [47, 48]. Better therapeutic responses could be induced by not only inducing TH1 responses but also concurrently suppressing TH2 responses.

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Review Article

The Inhibitory Role of B7-H4 in Antitumor Immunity: Association with Cancer Progression and Survival

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B7-H4 is one of the most recently identified members of B7 superfamily of costimulatory molecules serving as an inhibitory modulator of T-cell response. B7-H4 is broadly expressed in human peripheral tissues and inducibly expressed in immune cells. The expression of B7-H4 has been observed in various types of human cancer tissues, and its soluble form has been detected in blood samples from cancer patients. However, its precise physiological role is still elusive, as its receptor has not been identified and the expression levels are not consistent. This paper summarizes the pertinent data on the inhibitory role of B7-H4 in antitumor immunity and its association with cancer progression and survival in human patients. The paper also discusses the clinical significance of investigating B7-H4 as potential markers for cancer diagnosis and prognosis, and as therapeutic targets.

1. Introduction

Activation of T lymphocytes requires two independent but mandatory signals. The first signal requires recognition of the major histocompatibility complex (MHC)/antigen on antigen presenting cells (APCs) and corresponding antigen-specific T-cell receptor (TCR) on T cells. On the other hand, the second signal is delivered by the binding of costimulatory molecules and their receptors/ligands. In the absence of the costimulatory signal, the ligation of TCR with the MHC/antigen complex results in disfunction or anergy of T cells. The typical costimulatory signals are rendered by the molecules of the "classic" B7 family including CD80, CD86, and their receptor CD28 and cytotoxic T lymphocyte antigen (CTL-4), which could provide positive and/or negative costimulatory signals in initiating T-cell response. Recently, several B7 homologues have been identified, including B7-H1, B7DC, B7-H2, B7-H3, B7-H4, and B7-H6. B7-H4 (also known as B7x or B7S1) is among the most recently identified members of the B7 superfamily. It is broadly expressed in many human tissues and cells, and is shown to regulate adaptive immune response by inhibiting the proliferation, activation, and cytokine production of T cells, and host innate immune response by suppressing growth of neutrophil progenitors. It is also expressed in many types of human cancers, and has been used as a negative prognostic indicator for many human tumors. Therefore, B7-H4 represents a novel frontier of investigation for understanding the molecular regulation of the immune system and targeting B7-H4 may help to overcome the inhibitory immune network in tumor environments. This paper discusses the inhibitory role of B7-H4 in antitumor immunity, and its association with cancer progression and survival in human patients. It also discusses the clinical significance of investigating B7-H4 as potential markers for cancer diagnosis, and prognosis, and as therapeutic targets.

2. Structure and Expression Pattern of B7-H4

B7-H4 was identified by DNA sequence homology with other molecules of the B7 family in 2003 by three laboratories, which designated three different names to the same molecule, that is, B7S1, B7-H4, and B7x, respectively [1–3], but now B7-H4 has been most widely used. B7-H4 is a

type I transmembrane protein and has 20–30% amino acid homology in the extracellular portion with other B7 family members. The mouse and human amino acid sequences of B7-H4 share approximately 87% amino acid identity [2]. B7-H4 mRNA is widely expressed in human peripheral tissues, including lung, testis, pancreas, prostate, placenta, uterus, skin, muscle, intestine, stomach, kidney, liver, heart, brain, and ovary [1–3]. However, its protein expression on tissues seems to be limited [2, 4]. Initially, B7-H4 expression was observed in cancer cells of colon, prostate, lung, and fibrosarcoma [3, 5], and human ovarian and lung cancer tissues [4]. Subsequent studies from different laboratories have demonstrated that the expression of B7-H4 mRNA and protein was detected in all of the 23 melanoma cell lines [6], 5 gastric cancer cell lines [7], and 6 non-small-cell lung cancer cell lines [8]. To date, B7-H4 expression has been found in many different types of human cancer tissues, and soluble B7-H4 has also been detected in blood samples from cancer patients. The expression pattern of B7-H4 in human cancer tissues and its clinical significances will be discussed in Section 4.

B7-H4 is not expressed in naïve T and B cells, but after stimulation by interleukin−6 (IL-6) and IL−10, B7-H4 is inducibly expressed in APCs, including dendritic cells (DCs), monocytes and macrophages [1–3]. On the other hand, the DC−differentiation cytokines, granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4, decrease the expression of B7-H4 in these cells [9–11]. However, interferons (INFs) appear to have minimal effects on the induction of B7-H4 expression [9–11].

In human ovarian cancer, tumor-associated regulatory T (Treg) cells trigger macrophages to produce IL-6 and IL−10, and these cytokines in turn stimulate APCs to express B7-H4 in an autocrine and/or paracrine manner [9]. High levels of IL-6 and IL-10, but not GM-CSF and IL-4, are detected in the ovarian tumor microenvironment. Therefore, this dysfunctional cytokine network in the tumor microenvironment may enable APCs to express B7-H4. Interestingly, IL-4, IL-6, IL-10 and GM-CSF have no regulatory effects on the expression of B7-H4 on tumor cells, indicating that the expression of B7-H4 in tumor cells may be functionally distinct and differently regulated compared with APCs [9, 12].

To date, the receptor of B7-H4 has not yet been identified. B and T lymphocyte attenuator (BTLA) was initially proposed to be the receptor for B7-H4 [5], but further studies have not supported this proposal, as BTLA has not shown to directly bind to B7-H4 but may influence the appearance of an unknown receptor for B7-H4 on the Th1 cell surface [13–15].

3. Negative Effects of B7-H4 on Antitumor Immunity

3.1. Adaptive Immunity. B7-H4 inhibits the activation, proliferation, clonal expansion of $CD4^+$ and $CD8^+$ T-cells, thus suppressing the production of cytokines (IL-2, IFN-), and generation of alloreactive cytotoxic T lymphocytes (CTLs) by arresting the cell cycle in an *in vitro* T-cell activation assay [1, 2, 5]. B7-H4 expressed on the surface of surrogate APCs also inhibits the proliferation of T cells [2, 5]. *In vivo* blockade of endogenous B7-H4 by a specific mAb promoted T-cell response, indicating that B7-H4 plays an inhibitory role in T-cell activation [2]. The inhibitory effects of B7-H4 on T-cell activation and proliferation are also supported by the finding that acute lymphopenia-induced homeostatic proliferation of T cells promotes antitumor immunity. However, these cells display a severe deficit in the expression of B7-H4 as they show lower suppression by a specific Ab against B7-H4 and fail to produce IL-10 [16]. B7-H4-deficient Balb/c mice mounted mildly augmented Th1 responses and displayed slightly lower parasite burdens upon Leishmania major infection compared to the wild-type mice, indicating that B7-H4 could inhibit Th1 response against infection [17]. However, the lack of B7-H4 did not affect hypersensitive inflammatory responses in the airway or skin that are induced by either Th1 or Th2 cells. Likewise, B7-H4-deficient mice developed normal CTL reaction against viral infection [17]. These results suggest that B7-H4 may be one of multiple negative cosignaling molecules that collectively provide a fine-tuning mechanism for T-cell-mediated immune responses [18].

There is no direct evidence of a barrier function for B7-H4, although it is variably glycosylated in tumor-specific patterns, suggesting that glycosylation may be a potential mechanism for modulating interaction of CTLs with tumor cells [19]. A physical blockade would complement the ability of B7-H4, when ligated to its unknown receptor on T cells, to inhibit cytokine secretion, and proliferation of T cells predominantly through cell cycle arrest [2].

The effects of B7-H4 on B cells have not been investigated. However, enhanced B7-H4 expression on B cells infected with Epstein-Barr virus (EBV) increased the levels of intracellular reactive oxygen species (ROS), induced the expression of Fas ligand, and subsequently led to Fas-mediated and caspase-dependent apoptosis in association with increased release of cytochrome c, apoptosis-inducing factor (AIF), and EndoG from the mitochondria [20]. In a subsequent study by the same group, engagement of B7-H4 significantly reduced cell growth of EBV-positive lymphoma cells, resulting in cell cycle arrest at G0-G1 phase via downregulation of CDK4/6, CDK2, cyclin E/D expression, phosphor-AKT, and phosphor-cyclin E and upregulation of p21 expression [21]. These results suggest that B7-H4 may be a potential target for EBV-positive lymphoma therapy. Although not investigated, these studies may also imply that B7-H4 could inhibit proliferation and activation, and induce apoptosis of B cells, thus impairing the production of immunoglobulins and contributing to the suppression of adaptive immunity.

In addition, it has been demonstrated that tumoral $B7\text{-}H4^+$ macrophages and $CD4^+CD25^+FoxP3^+$ Treg cells suppressed tumor-associated antigen-specific T-cell immunity [10]. The tumor-associated macrophages spontaneously produce chemokine CCL22 to mediate Treg cell trafficking into tumors, and Treg cells induce the expression of B7-H4 on APCs and macrophages [10]. It has been shown that Treg cells induced macrophages to spontaneously produce IL-10

and IL-6, which in turn stimulated B7-H4 expression on macrophages in an autocrine manner through IL-10 and IL-6 [9]. The two studies suggest that Treg cells may convey their suppressive activity to APCs through B7-H4 induction [9].

3.2. Innate Immunity. To date, there has been only one published study [22], which has investigated the role of B7-H4 in innate immunity. It has been shown that the inhibitory effect of B7-H4 on innate immunity was mediated through controlling the growth of neutrophils [22]. B7-H4 knockout mice were more resistant to infection by Listeria monocytogenes than their littermates, suggesting that B7-H4 plays an inhibitory role on innate immunity. Further studies have shown that more neutrophils were observed in peripheral organs of B7-H4 knockout mice than their littermates but their bactericidal functions remained unchanged. *In vitro*, B7-H4 inhibited the growth of bone marrow-derived neutrophil progenitors, suggesting an inhibitory function of B7-H4 in neutrophil expansion. As augmented innate resistance is completely dependent on neutrophils, even in the absence of adaptive immunity, the results indicate that B7-H4 serves as a negative regulator of the neutrophil response to infection, and provides a new target for manipulation of innate immunity.

3.3. Cancer Immunity. B7-H4 has been found to be expressed at the mRNA and protein levels in many types of human cancers and negatively correlate with poor prognosis (Refer to Section 4). Expression of B7-H4 in human tumors is most likely due to aberrant regulation of posttranscription in tumors, since its cell surface protein expression is rare in normal human tissues, though abundant B7-H4 mRNA is detected [18]. B7-H4 was preferentially expressed in nondividing tumor cells from human gliomas and medulloblastomas, and in a subset of brain tumor stem-like $CD133^+$ cells [23]. The $CD133^+$ cell-initiated glioblastomas showed a higher proliferation index than $CD133^-$ cell-induced glioblastomas in immune-deficient mice [23].

Increased B7-H4 expression in tumor cells correlated with decreased cell apoptosis and enhanced outgrowth of tumors in several models, including the severe combined immunodeficiency (SCID)/Beige xenograft outgrowth model [22]. B7-H4 has also been shown to be extensively and variably N-glycosylated, which may serve as a "barrier" mechanism to evade immunosurveillance [22]. As suggested by Yi and Chen, the role of B7-H4 in tumor progression may be to transform precancerous cells and then protect them from immunosurveillance [18]. In addition, one study has shown that overexpression of B7-H4 promoted tumorigenesis of ovarian cancer in immunodeficient mice by increased proliferation rate, cell adhesion, migration, and invasion [24], implying that B7-H4 might have a direct effect on tumorigenesis independent of immunity. In another study, overexpression of B7-H4 on normal cells resulted in malignant cellular transformation of epithelial cells, perhaps by protecting the pretransformed cells from apoptosis, as siRNA knockdown of B7-H4 on tumor cell lines *in vitro* led to increased apoptosis [19]. However, the direct effect of B7-H4 on tumorigenesis has been only demonstrated in the above two studies, thus the exact mechanisms need further investigation.

In the tumor microenvironment, in addition to tumor cells, tumor-infiltrating macrophages [9, 10] and endothelial cells of small blood vessels [12] have also been found to constitutively express B7-H4. B7-H4 was highly expressed in tumor-associated macrophages in the ascites of ovarian cancer patients and contributed to tumor progression [10]. B7-H4 blockade by antisense oligonucleotides restored the function of macrophages to stimulate T cells and led to tumor regression *in vivo* [10, 11].

4. B7-H4 Expression in Human Cancers and Its Significance

In the present paper, the clinical data in support of the possible function of B7-H4 in antitumor immunity come from 26 retrospective analyses on 13 types of human cancers including the most common ones, that is, cancers of ovary, esophagus, kidney, stomach, liver, lung, colon, pancreas, breast and prostate, and melanoma. All relevant studies on the expression of B7-H4 on human cancer tissues or levels of soluble B7-H4 in human blood samples and the clinical significance are summarized in Table 1 [4–9, 12, 19, 23, 25–41]. A negative correlation between B7-H4 expression and T-cell infiltration has been reported [25, 32, 34]. However, such correlation was not observed in a study on melanoma [6].

The expression of B7-H4 has been most widely studied in ovarian cancer. To date, ten studies have investigated the expression of B7-H4 in ovarian cancer tissues and/or the level of soluble B7-H4 in blood samples from the ovarian cancer patients [4, 9, 19, 23, 28–30, 35–38]. The positive B7-H4 expression rates in ovarian cancer tissues range from 9 to 100% as shown by immunohistochemistry. Most of the studies have revealed the correlation between expression levels of B7-H4 and survival, pathological types, or tumor TNM staging. The levels of soluble B7-H4 in blood correlate with tumor stage, poor prognosis, and pathological types, indicating that B7-H4 may be a potential diagnostic marker and a prognostic predictor for ovarian cancer. However, one study did not show the similar correlation between soluble B7-H4 levels in blood and other diagnostic markers for ovarian cancer patients [27].

Breast cancer is the second most studied cancer for B7-H4 expression. To date, six studies have investigated the expression of B7-H4 in human breast cancer tissues [19, 30, 32, 33, 37], but two of which lack detailed data. In one study, 193 primary breast tumors and 246 metastatic breast tumors were examined by immunohistochemistry and the B7-H4 positive expression rates were as high as 95.4% in primary tumors and 97.6% in metastatic tumors, and the increased expression of B7-H4 correlated with negative progesterone receptor and HER-2/neu status [33]. Similarly, the other two studies have demonstrated a positive expression rate of B7-H4 mRNA and protein at 100%, determined by reverse transcription polymerase chain reaction (RT-PCR) [41] and immunohistochemistry [19], respectively.

Table 1: Relevant clinical studies investigating B7-H4 in samples from human cancer patients.

Author [ref.]	Journal	Year	Type of cancer	No. of samples	Methods	Positive rate (%)	Significances
Chen et al. [25]	Cancer Immunol Immunother	2011	Esophageal squamous cell carcinoma	112	IHC	95.5%	Correlation with gender, distant metastasis, TNM stage; reverse correlation with densities of $CD3^+$ and $CD8^+$ T cells, and survival
Jung et al. [26]	Korean J Urol	2011	Renal cell carcinoma	102	IHC	17.6% (early-stage T1)	No correlation with age, gender, TNM stage, lymphovascular invasion or nuclear grade; correlation with recurrence; reverse correlation with survival
Quandt et al. [6]	Clin Cancer Res	2011	Melanoma	29	IHC	96.6% (primary), 89.7% (metastatic)	Reverse correlation with survival; no correlation with $CD8^+$ T-cell infiltration
Arigami et al. [7]	J Surg Oncol	2010	Gastric cancer	94	RT-PCR	75.5%	Reverse correlation with survival
Jiang et al. [27]	Cancer Immunol Immunother	2010	Gastric cancer	156	IHC	44.9%	Reverse correlation with survival
Anderson et al. [28]	J Natl Cancer Inst	2010	Ovarian cancer	34	ELISA	—	No correlation with diagnosis markers
Qian et al. [30]	Clin Exp Med	2010	11 types of cancer*	289	IHC	Overall 52.9% (details refer to the article)	Correlation with stages
Yee et al. [40]	Histopathology	2010	Brenner tumor	34	IHC	100%	Higher proportion of expression than CA-125 and CEA
Oikonomopoulou et al. [29]	Br J Cancer	2008	Ovarian cancer	98	ELISA	—	Useful in predicting short-term (1-year) survival, time to progression after chemotherapy
Awadallah et al. [31]	Pancreas	2008	Pancreatic ductal adenocarcinoma	36	IHC	91.7%	More powerful than p53; potential diagnostic use
Thompson RH et al. [39]	Cancer Res	2008	Renal cell carcinoma	101	ELISA	52.5%	Correlation with stage; a potential serum marker for diagnosis and prognosis
Mugler et al. [32]	Appl Immunohistochem Mol Morphol	2007	Breast cancer	—	IHC	—	Correlation with invasive ductal carcinoma and reduced T-lymphocytes infiltration
Kryczek et al. [9]	Cancer Res	2007	Ovarian carcinoma	103	IHC	—	Correlation with Treg cell numbers

Table 1: Continued.

Author [ref.]	Journal	Year	Type of cancer	No. of samples	Methods	Positive rate (%)	Significances
Miyatake et al. [34]	Gynecol Oncol	2007	Uterine endometrioid adenocarcinoma	90	IHC, WB	100%	Correlation with high risk of uterine endometrioid adenocarcinoma; reverse correlation with T-cell infiltration
Simon et al. [35]	Gynecol Oncol	2007	Ovarian cancer	251	ELISA	48% (Stage I) 55% (Stage II) 67% (Stage III)	Correlation with poor prognosis
Simon et al. [36]	Gynecol Oncol	2007	Ovarian cancer	68	ELISA	100%	A promising marker for early detection of ovarian cancer
Zang X et al. [5]	Proc Natl Acad SCI USA	2007	Prostate cancer	823	IHC	99%	Associated with disease spread and poor outcome; an attractive targets for therapeutic manipulation
Sadun et al. [41]	Clin Cancer Res	2007	Breast and colorectal cancers	8 (breast), 11 (colorectal)	RT-PCR	100% (breast) Not consistent	A potential therapeutic target
Krambeck et al. [12]	Proc Natl Acad SCI USA	2006	Renal cell carcinoma	259	IHC	59.1%	B7-H4 is a useful prognostic marker for RRC patients
Sun et al. [8]	Lung Cancer	2006	Non-small-cell lung cancer	70	IHC	43%	Correlation with lower number of T cell infiltration
Simon et al. [37]	Cancer Res	2006	Colon, breast, lung, prostate, and ovarian cancers	1023 (confirmatory study: 200)	ELISA, IHC	—	Higher levels in endometrioid and serous histotypes than in mucinous histotypes in ovarian cancer
Tringler et al. [38]	Gynecol Oncol	2006	Ovarian cancer	125	IHC, WB	9% (mucinous), 100% (other histotypes and metastases)	A potential diagnostic marker or therapeutic target for ovarian cancer
Bignotti et al. [23]	Gynecol Oncol	2006	Ovarian serous papillary carcinoma	19	Microarray	—	Among the most highly overexpressed genes, indicating that B7-H4 is a candidate biomarker for early screening
Scalceda et al. [19]	Exp Cell Res	2005	Breast and ovarian cancers	19 (breast), 13 (ovarian)	RT-PCR, WB, IHC	100% (breast), 53.8% (ovarian)	A potential therapeutic target
Tringler et al. [33]	Clin Cancer Res	2005	Breast cancer	173 (primary), 246 (metastatic)	IHC	95.4% (primary), 97.6% (metastatic)	Correlation with negative progesterone receptor and HER-2/neu status, history of chemotherapy; no correlation with grade, stage
Choi et al. [4]	J Immunol	2003	Ovarian and lung cancers	22 (ovarian), 16 (lung)	IHC	85% (ovarian), 31% (lung)	A potential role in the evasion of tumor immunity

Notes: *Tumors from thyroid, esophagus, colon, pancreas, breast, liver, kidney, uterus, ovary, prostate and stomach. IHC: Immunohistochemistry; RT-PCR: Reverse transcription polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; IP: Immunoprecipitation; WB: Western blot analysis; CA-125: Cancer antigen-125; CEA: Carcinoembryonic antigen.

In two studies on lung cancer, 31% [4] and 43% [8] of lung cancer tissues were found to express B7-H4 detected by immunohistochemistry, respectively. In a study with 259 cases of renal cell carcinoma (RCC), 59.1% of the cancer tissues had B7-H4 protein expression [12]. However, the B7-H4 positive expression rate was found to be only 17.6% in 102 cases of early-stage RCC (T1), and B7-H4 expression did not correlate with age, gender, TNM stage, lymphovascular invasion, or nuclear grade, but correlated with cancer recurrence and negatively correlated with survival [26]. 75.5% (71/94) of gastric cancer tissues were found to express B7-H4 mRNA [7], but the B7-H4 protein positive expression rate detected by immunohistochemistry dropped to 44.9% in another study with 156 cases of gastric cancer [27]. In one study with 24 cases of gastric cancer, the positive rate was as low as only 12.5%. Although not widely investigated, over 90% of the tissues from melanoma [6], pancreatic ductal adenocarcinoma [31], uterine cancer [30, 34], esophageal squamous cell carcinoma [25], and prostate cancer [5] expressed B7-H4, shown by immunohistochemistry. Although one study has reported that 63.6% (14/22) of colon cancer tissues expressed B7-H4 [30], the expression of B7-H4 was found to be less consistent [37, 41]. B7-H4 was found to be expressed in 100% (34/34) of Brenner tumors [40]. Although Brenner tumors are of benign feature, this report is exceptionally included in Table 1.

Soluble B7-H4 was detected in blood samples from patients of ovarian cancer, RCC, colon cancer, breast cancer, lung cancer, and prostate cancer [8, 28, 29, 35–37, 39]. These studies indicate that serum B7-H4 may be a useful marker for diagnosis and prognosis, but the mechanism of production and the function of soluble B7-H4 remains unknown.

5. Potential of B7-H4 in Clinical Application

Because of the higher expression of B7-H4 in cancer tissues compared with corresponding normal tissues and its close correlation with stage, pathological types and biological behavior of tumors, and survival of cancer patients, we should pay attention to the potential diagnostic and prognostic capacities of B7-H4 for identifying cancer, determining pathologic variables, and predicting response to treatment and survival. We believe that B7-H4 could become potent tools to add to the oncologist's toolbox for early diagnosing cancer, monitoring the efficacy of treatments and predicting the prognosis. This may be possible when the expression patterns of B7-H4 have been investigated on a larger number of samples from different types of cancers and from multiple centers.

Given that B7-H4 is highly expressed in almost all the examined cell lines from cancers of colon, prostate, lung, and stomach, and fibrosarcoma and melanomas [3–8] and in various human cancer tissues (Table 1), it could be hypothesized that the expression of B7-H4 represents a mechanism of downregulating antitumor immunity, particularly T-cell response, at the level of the effector cells [5]. This paradigm calls for the development of new strategies for tumor immunotherapy by targeting B7-H4 [42]. B7-H4 inhibits T-cell function [1–3, 5], indicating that B7-H4-positive tumor cells have an advantage over the B7-H4-negative tumor cells by downregulating T-cell-mediated antitumor immunity. Consequently, the blockade of tumor-associated B7-H4 could offer a new therapeutic opportunity for enhancing antitumor immunity. Efficient neutralizing antibodies specific for human B7H4 are not yet available. Small interfering RNA (siRNA) [19] and antisense oligonucleotides specific for B7−H4 [10, 11] have been used to block B7−H4 expression. Blocking the expression of B7−H4 in tumor-associated macrophages disabled their suppressive capacity, enabled tumor-associated antigen- (TAA-) specific effector T cells function, and suppressed tumor growth in human ovarian cancer xenografts [10, 11]. In addition, the expression of B7-H4 on endothelial cells of tumor vasculature has also been observed in RCC tissues [12]. Although the mechanism accounting for what signals trigger B7-H4 expression in tumor vessels remains unknown, one most likely source could be the tumor microenvironments. Tumor blood vessels are distinct from normal resting blood vessels, and can be selectively destroyed without significantly affecting normal vessels. Therefore, blockade and/or destruction of tumor vasculature-associated B7-H4 might provide a dual beneficial therapy, that is, enhancement of T cell-mediated antitumor immunity and destruction of tumor vessels.

6. Conclusions and Future Prospects

Recent data indicate that B7-H4 functions in peripheral tissues to negatively regulate immune responses in target organs. While its broad distribution is observed at mRNA level, limited expression at the protein level suggests that tight control of B7-H4 is imposed at posttranscriptional level. Receptor identification remains the manifest topic and is critical for understanding the role of B7-H4, as it is certainly essential to understand the complex role, but continues to be difficult due primarily to low receptor/ligand affinities. Therefore, more studies are required to seek and identify the receptor for B7-H4. Increased B7-H4 expression in tumor tissues and high levels in blood samples of cancer patients represent a realistic opportunity to design novel immunotherapeutic approaches by regulating the immune response through manipulating the expression of B7-H4 and/or its receptor. B7-H4 can also serve as a useful biomarker for cancer diagnosis and prognosis prediction, when its expression patterns have been further investigated.

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Research Article

Involvement of Indoleamine 2,3-Dioxygenase in Impairing Tumor-Infiltrating CD8$^+$ T-Cell Functions in Esophageal Squamous Cell Carcinoma

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The indoleamine 2,3-dioxygenase-(IDO-) mediated microenvironment plays an important role in tumor immune escape. However, the inhibitory effects of IDO on the CD8$^+$ tumour-infiltrating lymphocytes (CD8$^+$ TILs) in esophageal squamous cell carcinoma (ESCC) have not been clarified yet. Here, we found that the level of IDO expression in ESCC tumor specimens correlated with a reduction in the number of CD8$^+$ TILs. Patients with high IDO expression and a low number of CD8$^+$ TILs had significantly impaired overall survival time. IDO expression and functional enzyme activity in ESCC cell lines could be induced by IFNγ. When exposed to the milieu generated by IDO-expressing Eca109 cells, the CD8$^+$ TILs were suppressed in proliferation, and their cytolytic functions against target tumor cells were lost. These results suggested that impairing CD8$^+$ TIL functions by IDO expressed in ESCC possibly contributed to the finding that patients with higher IDO expression have more aggressive disease progression and shorter overall survival time.

1. Introduction

Effector CD8$^+$ tumor-infiltrating lymphocytes (CD8$^+$ TILs) are major mediators for host's antitumor immunity [1–5]. Increasing studies have reported that higher numbers of CD8$^+$ TILs within either esophageal squamous cell carcinoma (ESCC) epithelium or stroma have a better prognosis [6, 7]. Recently, several reports showed that tumoral indoleamine 2,3-dioxygenase (IDO) expression correlated with a reduced number of CD8$^+$ or CD3$^+$ TILs in colorectal cancer, ovarian cancer, and endometrial cancer, possibly contributing to disease progression and impaired clinical outcome [8–10].

IDO is responsible for initiating the first rate-limiting step in tryptophan (Trp) metabolism in the kynurenine (Kyn) pathway [11, 12]. Growing evidence suggests that various types of human tumor cells express IDO, and inflammatory mediators, especially interferon-γ (IFNγ), have the specific ability to induce IDO expression [13, 14]. Tumoral IDO expression has been shown to correlate with poor clinical prognosis in ovarian carcinoma, endometrial carcinoma, lung cancer, osteosarcoma, and colon carcinoma [8, 15–18]. IDO-mediated Trp metabolism in antigen-presenting cells and tumor cells represents a vital mechanism for potential T-cell suppression during tumor growth [19–21]. In the experimental rat lung allograft model, IDO not only reduced the number of CD8$^+$ T cell infiltration but also impaired the cytotoxic function of effector CD8$^+$ T-cells, this impairment was responsible for the IDO-dependent immune suppression [22]. Our previous study also showed

that exposure to the milieu created by an IDO-positive nasopharyngeal carcinoma cell line significantly impaired the lymphocyte cytotoxicity against target tumor cells [23].

A previous study on a small group of ESCC patients showed that IDO mRNA was expressed in ESCC tumor specimens, and ESCC patients with higher levels of IDO mRNA expression had a worse survival rate than those with lower levels of IDO mRNA expression [24]. In contrast, Liu reported that the level of IDO expression did not correlate with the clinic outcomes of ESCC patients [25]. In the current study, we investigated the relationship between IDO expression and the degree of tumor infiltration of $CD8^+$ T cells, and the clinical significance of IDO expression in ESCC. We also explored the effect of IDO on the proliferation and function of $CD8^+$ TILs in ESCC.

2. Materials and Methods

2.1. Patients. A total of 135 ESCC samples were histologically and clinically diagnosed when the patients with primary ESCC underwent radical esophagectomy between 2001 and 2004 at the Cancer Center of Sun Yat-sen University. No patients had received prior anticancer treatment. Prior to the use of these clinical materials for investigation, informed consent from patients and approval from the Institute Research Ethics Committee were obtained. The clinical typing of the tumors was determined according to the pathological TNM classification [26]. Clinical information of the samples is described in detail in Table 1. The numbers undergoing metastasis pertain to the presence of metastasis at any time during follow-up. The median followup time for overall survival was 49.0 months for patients still alive at the time of analysis, and the time ranged from 7 to 78 months. A total of 91 (67.4%) patients died during followup.

2.2. Immunohistochemistry and Immunoblotting. Immunohistochemistry and western blot were performed as described previously [23, 27]. For immunohistochemistry, an anti-IDO polyclonal antibody (1 : 500, generated in our laboratory [23]) and a mouse monoclonal anti-CD8 (1 : 150, BD Pharmingen) were incubated with the tissue sections overnight at 4°C. For negative controls, the primary antibody was replaced by normal rabbit or mouse serum. After washing, tissue sections were treated with biotinylated anti-mouse or anti-rabbit antibody (Zymed), followed by further incubation with streptavidin HRP complex. For Western blot analysis, IDO was detected using an anti-IDO polyclonal antibody (1 : 5,000). An anti-β-actin monoclonal antibody (1 : 5,000) was used to confirm equal loading.

2.3. Scoring of IDO Expression in Tumor Cells. The degree of immunostaining was reviewed and scored by two independent observers, as described previously [28]. According to the percent of positive cells, one score was given for each as follows: <5% of the cells = 1 point; 6–35% of the cells = 2 points; 36–70% of the cells = 3 points; >70% of the cells = 4 points. Another score was given according to the intensity of staining as follows: negative staining = 1 point; weak staining (light yellow) = 2 points; moderate staining (yellowish brown) = 3 points; strong staining (brown) = 4 points. A final score was then calculated by multiplying the above two scores. If the final score was ≥4, the tumor was considered high expression; otherwise, the tumor was considered low expression. IDO expression in tumor stromal cells was not considered because IDO immunostaining on nontumor cells was not remarkable in all cases examined.

2.4. Quantification of TIL Cells within ESCC. $CD8^+$ TILs were classified into two groups by their localization: (a) intraepithelial, cells infiltrating into the tumor epithelium; (b) stromal, cells infiltrating the tumor stroma adjacent to cancer epithelia or the stroma along the invasive margin of the cancer epithelia. Quantification of $CD8^+$ TILs was done according to the previous reports of Cho and Schumacher with some modifications [6, 7]. Three independent areas with the most abundant $CD8^+$ TIL infiltration were selected, and the intraepithelial $CD8^+$ TILs and stromal $CD8^+$ TILs were independently counted in each microscopic field at $200 \times (0.0625\,mm^2)$. The average count for three areas was accepted as the number of $CD8^+$ TILs in each case. We classified patients into two groups by intraepithelial $CD8^+$ TIL counts: the high intraepithelial $CD8^+$ TIL group (mean ≥ 10) and low intraepithelial $CD8^+$TIL group (mean < 10). On the basis of stromal $CD8^+$TIL counts, patients were classified into two groups in the same manner: the high stromal $CD8^+$ group (mean ≥ 20) and low stromal $CD8^+$ group (mean < 20).

2.5. ESCC Cell Culture and $CD8^+$ T-Cell Isolation. The ESCC cell lines Eca109, TE-1, and KYSE140 (Cell Bank of Type Culture Collection of Chinese Academy of Sciences) were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum. IFNγ (China National Biotec Group) was added to the medium at 0–500 U/mL, for the indicated time. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of patients with ESCC before surgical treatment by Ficoll density gradient (Sigma-Aldrich). For the isolation of TILs, fresh tumor tissues from ESCC patients who underwent surgical treatment at our hospital were finely minced and subjected to enzymatic digestion. The resultant suspensions were filtered through a 25 μm mesh filter, and the single-cell filtrate was washed twice in PBS followed by Ficoll/Hypaque purification. The isolation of $CD8^+$ T lymphocytes from PBMCs and TILs was performed by means of immunomagnetic beads using a Dynal CD8 positive isolation kit (Invitrogen Dynal). Purity of $CD8^+$ was >98% $CD8^+$ as checked by flow cytometry and $CD8^+$ T cells were grown in complete RPMI 1640 medium.

2.6. Measurement of IDO Activity. Trp and Kyn concentrations were analyzed by reverse-phase high-performance liquid chromatography (HPLC; Waters) as described previously [23]. IDO activity was determined by the Kyn to Trp ratio (Kyn/Trp, μM/μM).

2.7. Cell Proliferation, Apoptosis, and Cytotoxicity Assay. Eca109 cells were cultured in 6-well plates (3×10^5 cells/well)

Table 1: Characteristics of 135 patients with esophageal squamous cell carcinoma and correlation between the clinicopathologic features and expression of IDO.

Characteristics	All cases (n)	IDO low n (%)	IDO high n (%)	Significance (P)*
Gender				
Male	100	45 (45.0)	55 (55.0)	0.069
Female	35	22 (62.9)	13 (37.1)	
Age (y)				
<60	80	35 (43.8)	45 (56.2)	0.100
≥60	55	32 (58.2)	23 (41.8)	
Stage				
I-II	74	45 (60.8)	29 (39.2)	0.004
III-IV	61	22 (36.1)	39 (63.9)	
Histological differentiation				
Well	41	25 (61.0)	16 (39.0)	0.075
Moderate	57	22 (38.6)	35 (61.4)	
Poor	37	20 (54.1)	17 (45.9)	
Tumor diameter				
<40mm	54	25 (46.3)	29 (53.7)	0.527
≥40mm	81	42 (51.9)	39 (48.1)	
Depth of invasion				
Submucosa	7	5 (71.4)	2 (28.6)	0.300
Muscularis propria	46	25 (54.3)	21 (45.7)	
Adventitia	82	37 (45.1)	45 (54.9)	
pT classification				
T1-T2	44	28 (63.6)	16 (36.4)	0.024
T3-T4	91	39 (42.9)	52 (57.1)	
pN classification				
Yes	65	25 (38.5)	40 (61.5)	0.012
No	70	42 (60.0)	28 (40.0)	
P metastasis				
Yes	9	4 (44.4)	5 (55.6)	0.982
No	126	63 (50.0)	63 (50.0)	
Intraepithelial $CD8^+$				
High (≥10)	62	38 (61.3)	24 (38.7)	0.013
Low (<10)	73	29 (39.7)	44 (60.3)	
Stromal CD8+				
High (≥20)	66	42 (63.6)	24 (36.4)	0.001
Low (<20)	69	25 (36.2)	44 (63.8)	

in the absence or presence of 50-U/mL IFNγ for 12 hr, and the medium was then replaced by fresh medium with or without 100 μM 1-methyl-L-tryptophan (1 MT, Sigma-Aldrich). Then, 24 hr after medium replacement, the culture media were harvested as Eca109-conditioned media (Eca109-CMs). $CD8^+$ T-cell proliferation was assessed by standard thymidine incorporation assay as described previously [29]. Briefly, 1×10^5 $CD8^+$ T cells were cultured in Eca109-CMs and stimulated with plate-bound anti-CD3 mAb (OKT3, ATCC) and soluble anti-CD28 mAb (BD Bioscience). After 72 hours of culture, 1-μCi ^{3}[H] thymidine was added, and incorporation was measured after 24 hr in a β-Counter (Wallac). The proportion of apoptotic cells from different culture conditions were examined by flow cytometry using an Annexin V-FITC apoptosis detection kit (Beckman Coulter).

The cytotoxic activity of $CD8^+$ T cells was determined by a standard lactate dehydrogenase (LDH) release assay using the CytoTox 96 (Promega) as previously described [23]. Briefly, the Eca109 cells (5×10^3 cells/well) were cultured as target cells. IL-2-stimulated $CD8^+$ T cells were incubated in different Eca109-CMs as the treated effector cells. The target cells and effector cells suspensions were cocultured at various indicated effector : target (E/T) ratios. After 4 h of incubation, the release of LDH into the supernatant was quantified by recording the absorbance at 490 nm. The

percentage of cytotoxicity was calculated as manufacturer described.

2.8. Statistical Analysis. All statistical analyses were conducted using the SPSS 16.0 statistical software package. Parametrically distributed data are presented as mean ± SD. Comparison of the number of $CD8^+$ TILs in the IDO-low group and IDO-high group was performed with the Mann-Whitney U test. The Pearson χ^2 and Fisher's exact test were used to analyze the relationship between IDO expression and clinicopathologic characteristics or the number of $CD8^+$ TILs. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. Comparison between paired or unpaired groups was performed using the appropriate Student's t-test. A P value of < 0.05 in all cases was considered statistically significant.

3. Results

3.1. Expression of IDO in Archival Esophageal Tumor Tissues and Association of IDO Expression with $CD8^+$ TILs. IDO protein was detected in all 135 paraffin-embedded archived ESCC tissues (100%) by immunohistochemistry. IDO immunoreactivity was observed at various levels, and localization was observed in the cytoplasm of the tumor cells. By visual estimation, tumors were grouped into two categories: "IDO-high expression" and "IDO-low expression" according to a proportion and intensity score described in Methods (Figures 1(a)–1(d)). IDO was highly expressed in 68 of 135 (50.4%) tumor tissues, whereas 67 of 135 (49.6%) cases showed low IDO expression levels (Table 1). In contrast, in the normal esophageal tissue adjacent to cancers, IDO had absent to weak staining patterns (Figures 1(e)–1(f)).

To investigate the relationship between IDO expression and the $CD8^+$ TIL population, we evaluated the number of $CD8^+$ TIL infiltrating into the tumor epithelium or stroma (Figures 1(g)–1(j)). The number of intraepithelial $CD8^+$ TILs in IDO-high expressing tumors (range 1–22; median10.6) was significantly lower than in IDO-low expressing tumors (range 6–37; median 21.1; $P = 0.013$; Figure 2(a)). Similarly, IDO-high expressing tumors exhibited a significantly lower proportion of stromal $CD8^+$ TILs (range 7–36; median 20.1) compared with IDO-low expressing tumors (range 18–62; median 41.2; $P = 0.001$; Figure 2(b)). The correlation of IDO expression and $CD8^+$ TIL counts is summarized in Table 1. These results suggest that the level of IDO expression is inversely correlated with the number of intraepithelial $CD8^+$ TILs and the number of stromal $CD8^+$ TILs.

3.2. Association of IDO Expression with Clinicopathological Variables. As shown in Table 1, there was no significant correlation between the expression level of IDO protein and gender, age, histological classification, histological differentiation, tumor diameter, depth of invasion, and distant metastasis of esophageal cancer patients. However, the expression of IDO is closely associated with stage of esophageal cancer patients ($P = 0.004$), T classification ($P = 0.024$), and pN classification ($P = 0.012$). Higher staging, higher T classification and lymph node metastasis correlated with higher IDO expression.

3.3. Impact of IDO Expression and $CD8^+$ TIL Counts on Patient Overall Survival. Overall survival analysis according to Kaplan-Meier analysis showed that although survival curves crossed at 73 months, the expression of IDO protein in esophageal carcinoma was significantly correlated with patients' survival time ($P = 0.041$), indicating that higher levels of IDO expression was correlated with shorter survival time whereas the low-IDO expression group had better survival (Figure 3(a)). The median survival of patients with high IDO expression was much shorter (23 months) than those with low IDO expression (33 months).

Next, we analyzed the effect of the $CD8^+$ TIL counts on patient survival. Figure 3(b) showed that patients in the high intraepithelial $CD8^+$ TIL groups (≥ 10) showed a significantly higher survival time compared with those in the low intraepithelial $CD8^+$ TIL groups (<10; $P = 0.043$). Similarly, those in the high stromal $CD8^+$ TIL groups (≥ 20) exhibited a significantly higher survival time compared with those in the low stromal $CD8^+$ TIL groups (<20; $P = 0.024$; Figure 3(c)). These results indicated that patients with low intraepithelial and stromal $CD8^+$ TILs had significantly impaired survival compared with patients with high intraepithelial and stromal $CD8^+$ TILs.

3.4. IDO Expression in Esophageal Carcinoma Cell Lines and Induction by IFNγ. The effect of IFNγ on IDO expression was investigated in the ESCC cell lines: Eca109, TE-1, and Kyse140. As shown in Figure 4(a), the western blot assay showed that none of the cell lines constitutively expressed the IDO protein. IDO could be induced in these cell lines by treatment with 100-U/mL IFNγ. Among these cell lines, Eca109 had the highest expression level. Then, we performed western blot analysis to investigate the effects of varying concentrations of IFNγ on the expression of IDO in Eca109 cells. Figure 4(b) shows that treatment with low-dose IFNγ (10 U/mL) could induce IDO expression, which was further increased in an IFNγ concentration-dependent manner. The enzymatic activity of IDO was also investigated by HPLC (Figure 4(c)). The enzymatic activity of IDO was undetectable in the culture medium of untreated Eca109 cells, but the activity was observed with 10 U/mL IFNγ stimulation, peaking with 50 U/mL IFNγ, and then it remained at almost the same level with increasing IFNγ stimulation. Thus, consistent with the results of the western blot analysis, IDO expression in ESCC cell lines was an inducible event that was highly sensitive to IFNγ stimulation.

3.5. Exposure to the Microenvironment Created by IDO-Positive Eca109 Cells Severely Suppresses $CD8^+$ T Cells Proliferation and Does Not Induce $CD8^+$ T Cell Apoptosis. To address our above observations that the samples with a high IDO expression also had a low number of $CD8^+$ TILs, we tested whether the exposure to CM from IFNγ-treated Eca109 cells could inhibit $CD8^+$ T-cell proliferation and/or induce $CD8^+$ T-cell apoptosis. We treated Eca109 cells with or without

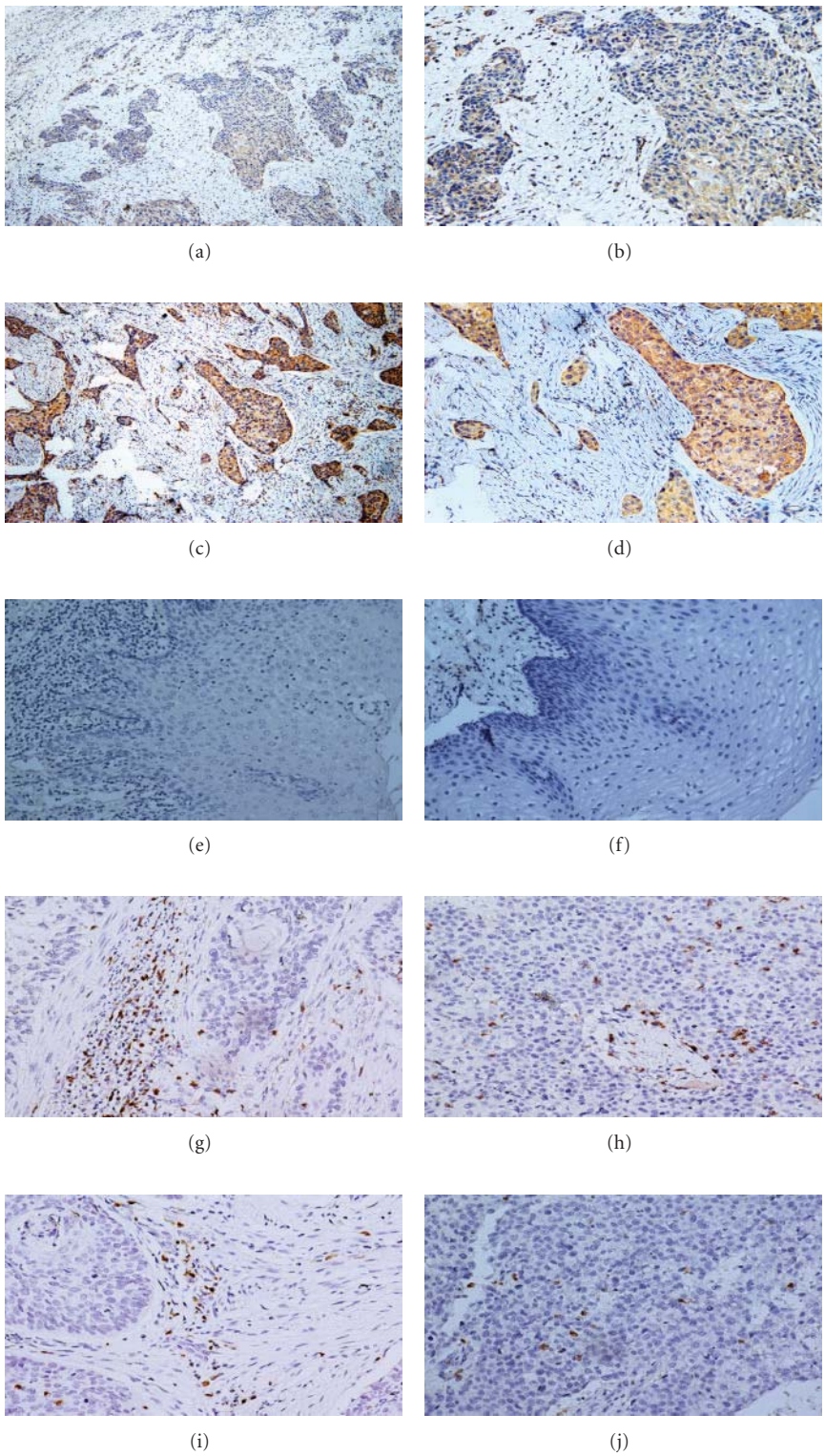

Figure 1: Expression analysis of IDO protein in ESCC by immunohistochemistry and representative immunohistochemical staining for $CD8^+$ TILs. (a, b) Example of IDO-low expression. (c, d) Example of IDO-high expression. (e, f) Staining of IDO in normal esophageal epithelial tissue, (e) absent; (f) weak. (g) Example of stromal $CD8^+$ TIL staining in IDO-low expression tumor tissue. (h) Example of intraepithelial $CD8^+$ TIL staining in IDO-low expression tumor tissue. (i) Example of stromal $CD8^+$ TIL staining in IDO-high expression tumor tissue. (j) Example of intraepithelial $CD8^+$ TIL staining in IDO-high expression tumor tissue (original magnification, (a) and (c) $\times 100$; (b, d, e–j) $\times 200$).

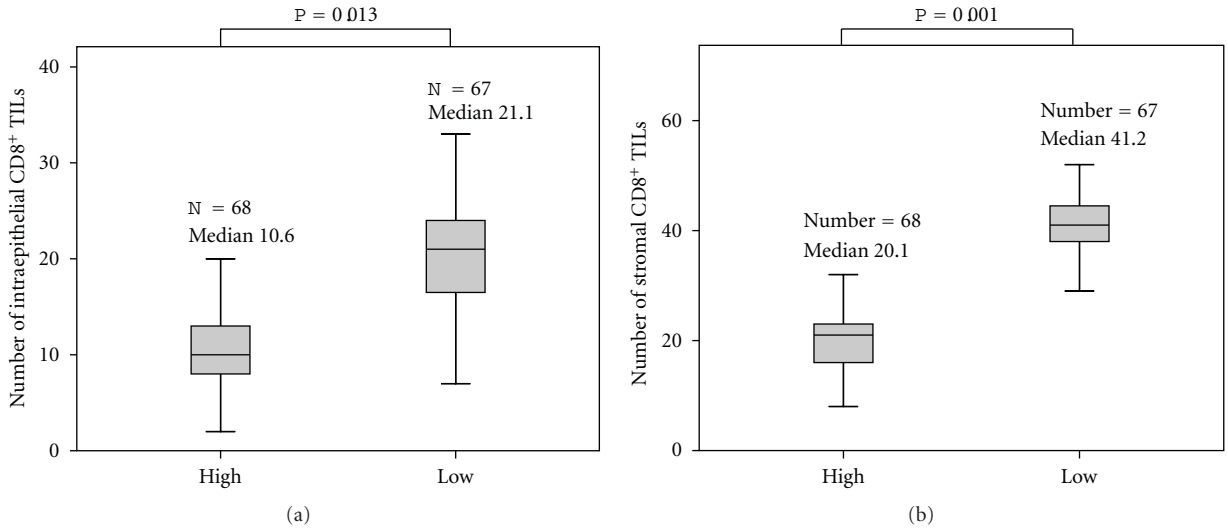

Figure 2: Association of IDO expression with the number of $CD8^+$ TILs. There was a significant difference in both the number of intraepithelial $CD8^+$ TILs (a) and stromal $CD8^+$ TILs (b) between the tissues with IDO-high expression and IDO-low expression.

IFNγ before $CD8^+$ T cells were cultured in these CMs under anti-CD3/CD28 mAb stimulation. $CD8^+$ T-cell proliferation both from PBMCs and TILs was significantly lower in treated media compared with the proliferation in untreated media (Figure 5(a)). These findings indicated that the CM derived from IFNγ–treated Eca109 cells suppressed $CD8^+$ T-cell proliferation. Next, we tested whether the observed effects of the CM on $CD8^+$ T-cell proliferation rates were related to functional IDO enzyme activity. As shown in Figure 5(b), IFNγ–treated Eca109 cells had IDO enzyme activity whereas untreated cells did not have IDO enzyme activity. To further demonstrate the effect of IDO on the proliferation of $CD8^+$T cells, the specific IDO inhibitor 1MT was used to block the enzyme activity. When exposed to IFNγ-treated Eca109-CM, the proliferation of $CD8^+$ T cells both from PBMCs and TILs was almost completely restored by the 1MT addition (Figure 5(a)), the functional IDO enzyme activity in IFNγ-treated Eca109-CM was dramatically inhibited by the 1 MT (Figure 5(b)). Taken together, the data indicated that the media created by IDO-positive Eca109 cells suppressed $CD8^+$ T-cell proliferation *in vitro*.

We next aimed to determine the effects of IDO on apoptosis of $CD8^+$ T cells. Without simulation, $CD8^+$ T cells from both PBMCs and TILs exhibited no significant difference in the frequency of apoptotic cells between the cells exposed to IFNγ-treated and untreated Eca109-CM. Similarly, under anti-CD3/CD28 simulation, there was no significant difference in the frequency of apoptotic cells between the cells exposed to IFNγ-treated and untreated Eca109-CM (Figure 5(c)). These results indicated that IDO derived from IFNγ-treated Eca109 cells exerted no significant impact on the apoptosis of $CD8^+$ T cells.

3.6. Exposure to Conditioned Medium Derived from IDO-Positive Eca109 Cells Impairs Cytolytic Activity of $CD8^+$ T Cells. To investigate whether IDO has an effect on the cytolytic activity of $CD8^+$ T cells, a standard LDH release assay was conducted using Eca109 cells as targets and $CD8^+$ T cells from PBMCs or from TILs stimulated by IL-2 as effectors. IL-2 activated $CD8^+$ T cells from both PBMCs and TILs lysed the target cells at different E/T ratios when exposed to CMs from untreated Eca109 cells whereas the lysis rate was remarkably reduced when the T cells were exposed to IFNγ-treated Eca109-CMs. But the cytolytic activity of $CD8^+$ T cells from both PBMCs and TILs was effectively restored when exposed to CM derived from IFNγ-treated Eca109 cells and in the presence of 1MT (Figures 6(a) and 6(b)). Because the IDO enzyme activity only presented in CM from IFNγ-treated Eca109 cells, but not in CM from untreated cells or dramatically reduced in CM from IFNγ-treated Eca109 cells with 1MT (Figure 6(c)). These results suggested that the inhibition on $CD8^+$ T cell-mediated cytotoxicity was attributed to IDO created by IFNγ-treated Eca109 cells, and 1MT can abrogate this effect, providing a potential role of IDO in impairing $CD8^+$ T-cell cytotoxic function.

4. Discussion

In this study, we confirmed previous reports that ESCC patients with higher numbers of $CD8^+$ TILs within either tumor epithelium or tumor stroma had a better prognosis than those with a lower number of $CD8^+$ TILs [6, 7]. Recent studies have suggested that tumoral IDO expression correlates with a reduced number of $CD8^+$ T-cell infiltration into tumor sites [8–10]. In line with these observations, we also found that IDO expression in ESCC was inversely correlated with the number of $CD8^+$ TILs both in tumor epithelium and tumor stroma. Moreover, our data indicated that the expression of IDO correlated with the poor clinical outcome of ESCC patients and are consistent with a previous study that ESCC patients with higher levels of IDO expression had

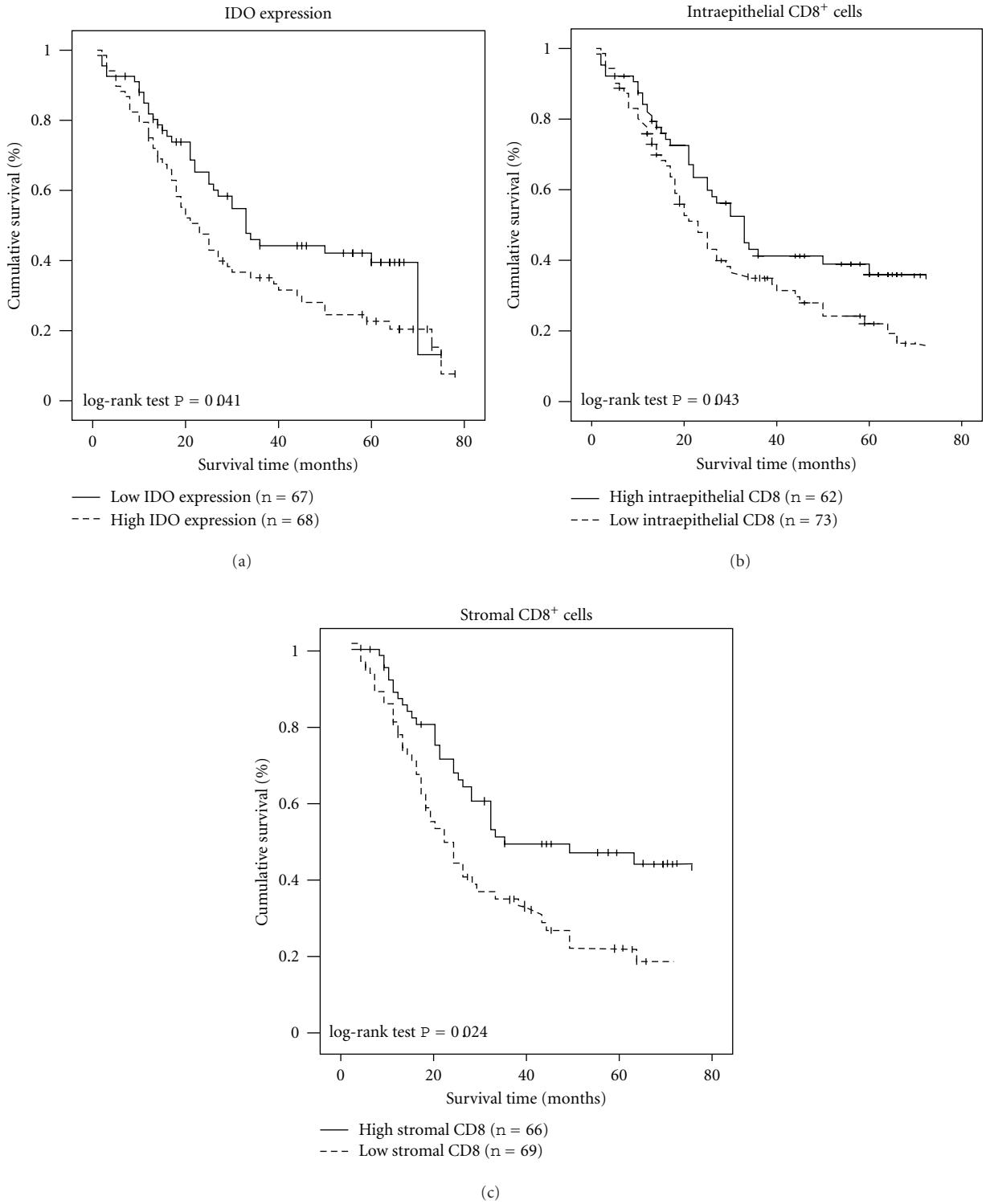

Figure 3: Overall survival curves drawn using Kaplan-Meier method according to the IDO expression, the number of intraepithelial $CD8^+$ TILs and the number of stromal $CD8^+$ TILs in 135 ESCC patients. (a) There was a significant difference in the overall survival between patients with low IDO expression (bold lines) and high IDO expression (dotted lines). (b) Significant differences in overall survival between the high intraepithelial $CD8^+$ TIL groups (≥ 10) and the low intraepithelial $CD8^+$ TIL groups (<10). (c) Significant differences in overall survival between the high stromal $CD8^+$ TIL groups (≥ 20) and the low stromal $CD8^+$ TIL groups (<20).

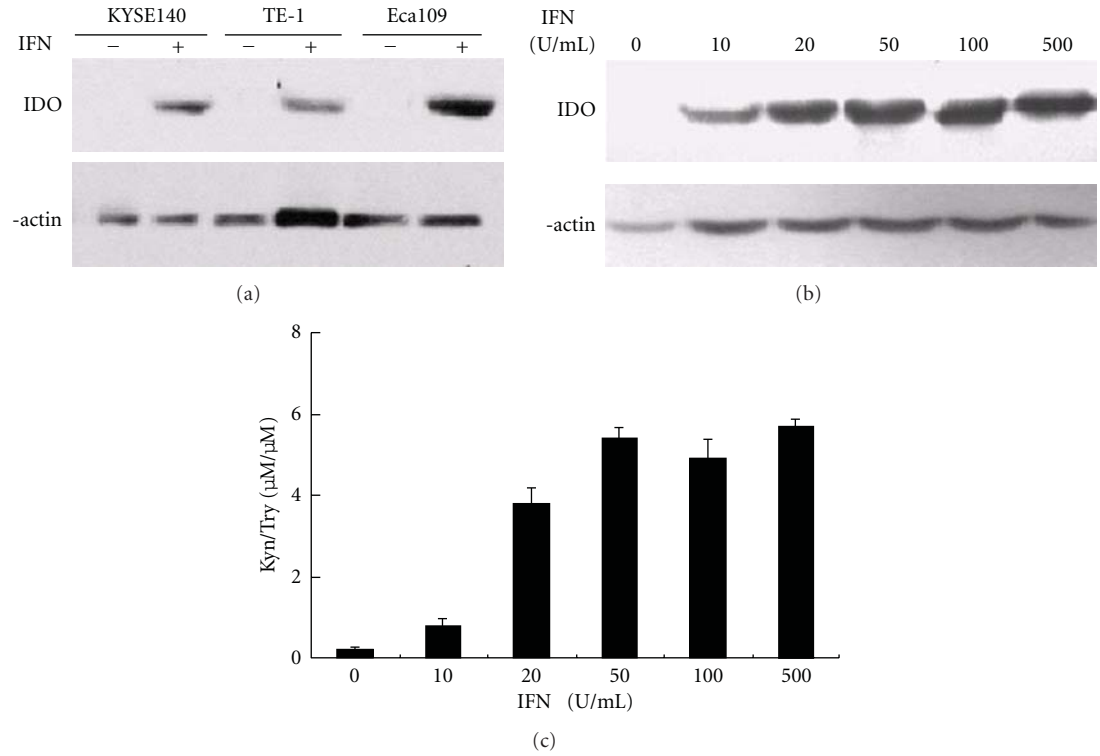

Figure 4: Effect of IFNγ on IDO expression in the esophageal carcinoma cell lines. Esophageal carcinoma cell lines Kyse 140, TE-1, and Eca109 were treated with or without 100 U/mL IFNγ for 24 hr, and expression of the IDO protein was assessed by western blot (a). IFNγ induced IDO protein expression in Eca109 cells in a dose-dependent manner. Eca109 cells were cultured in the presence of indicated concentrations of IFNγ for 24 hr, IDO expression was analyzed by western blotting (b); the supernatants of Eca109 cells were analyzed for Kyn and Trp production using HPLC (c). Columns, mean of three independent experiments. Bars, SD.

a worse survival rate than patients with lower levels of IDO expression [24]. However, these results were in contrast with those of Liu et al. in that IDO expression was not significantly correlated with clinical outcomes [25]. The less marked impact of IDO on clinical outcomes reported by Liu et al. might be due to the comparatively small patient population analyzed. Consistent with other studies that serum/plasma Kyn/Trp levels were used as indicator of IDO activity and were found higher IDO enzyme activity predicted worse survival of cancer patients [8, 30]. Our findings also suggest that higher tumoral IDO expression and lower numbers of $CD8^+$ TILs might contribute to a worse survival in ESCC patients.

IDO is widely distributed in mammals and is preferentially inducible by IFNγ [11]. There have been studies indicating that IDO is upregulated in many tumor cell lines only upon treatment with IFNγ and/or other inflammatory mediators [31, 32]. Recently, Godin-Ethier reported that activated T cells induce functional IDO expression in breast and kidney tumor cell lines and that this was partly attributable to IFNγ [13]. In the present study, an analysis of Kyn production demonstrated that IDO enzymatic activity was only present in the esophageal cancer cell lines treated with IFNγ, and western blot also confirmed the finding. Furthermore, we showed that IFNγ induced the IDO expression in Eca109 cells at concentrations as low as 10 U/mL, implying that IDO expression in Eca109 cells could be easily induced by low levels of IFNγ. The finding that ESCC tumor cell lines show no constitutive IDO expression and that only IFNγ was able to induce huge activity corresponds very well to early Werner-Felmayer's study [33], but is a little against claims made by Uyttenhove [12]. This discrepancy may be the different tumor cell lines used in different studies.

Tumor-associated antigen-presenting cells, such as macrophages and dendritic cells, and tumor-associated antigen-specific T-cells within the tumor microenvironment release this cytokine [34]; thus, they might induce IDO expression in esophageal tumor cells *in vivo*.

Once expressed in tumor cells, IDO degrades the essential amino acid Try to form N-formyl Kyn and produces a series of immunosuppressive Try metabolites [12]. Two apparently disparate mechanisms have been proposed to explain how IDO plays a role in immune suppression. One suggests that depleting local L-Try, an essential amino acid for T-cell proliferation, may block the cell cycle in the G1 phase and render T cells susceptible to proliferation arrest [12, 35, 36], and the other suggests that IDO may suppress T-cell responses by the action of Try metabolites, such as Kyn, which are toxic and proapoptotic for T cells [19]. Our data showed that exposure to the microenvironment created by IDO-positive Eca109 cells severely suppressed $CD8^+$ T-cell proliferation and did not significantly induce $CD8^+$ T-cell apoptosis, and the results favor the model describing the proliferation arrest of $CD8^+$ T cells. Thus, the inhibition

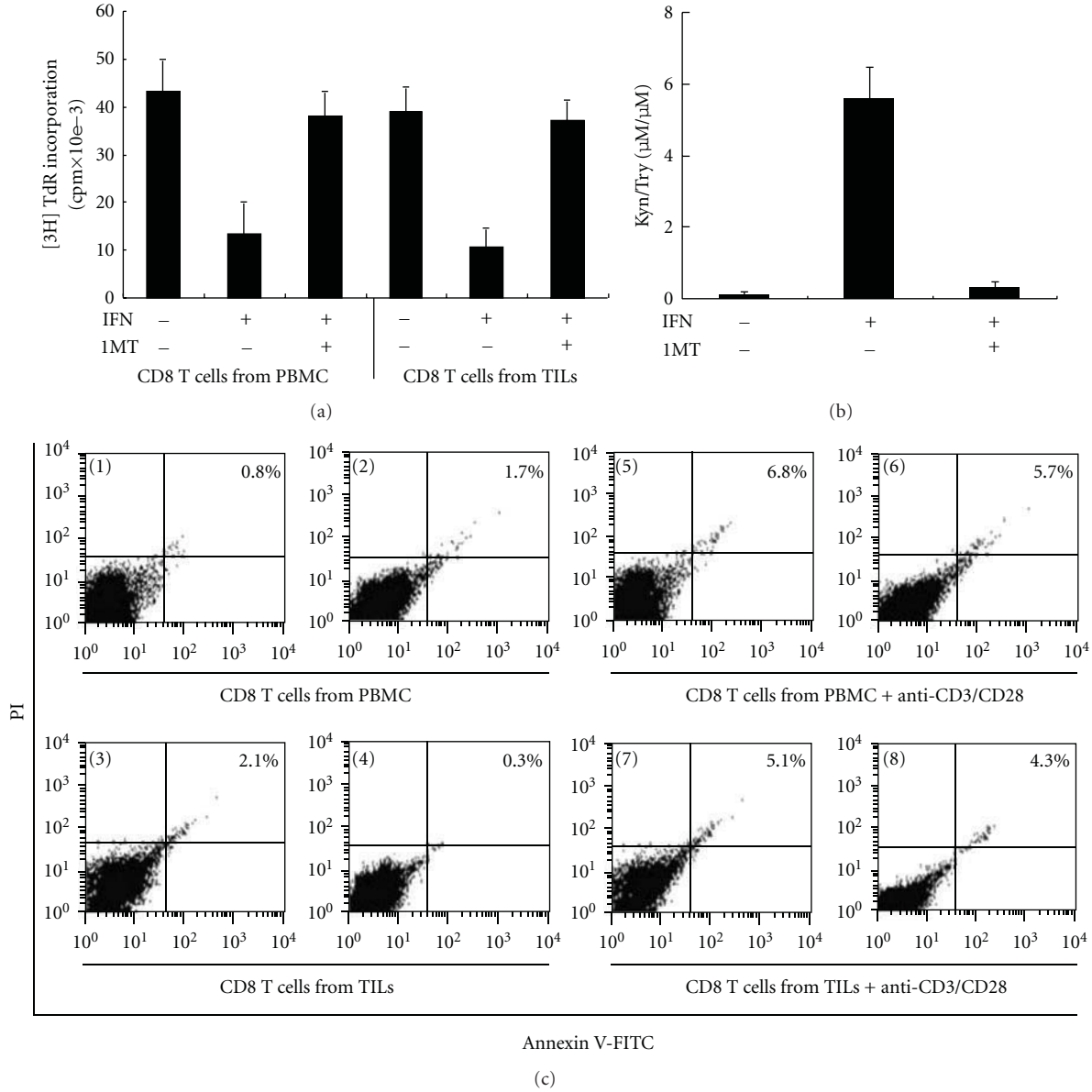

Figure 5: Effect of IDO on the proliferation and apoptosis of $CD8^+$ T cells from both PBMCs and TILs. (a) Proliferation of $CD8^+$ T cells from PBMCs or TILs was suppressed by IDO-expressing Eca109 cells. $CD8^+$ T cells were cultured in conditioned media and activated with anti-CD3/CD28 antibodies. Proliferation of $CD8^+$ T cells was assayed by [3H] TdR incorporation. (b) Concentrations of Kyn and Try were measured in the supernatants of Eca109 cells treated with or without IFNγ (50 U/mL) and/or 1MT (100 μM) for 24 hr. Kyn and Try productions were analyzed by HPLC. (c) Effect of IDO on the apoptosis of $CD8^+$ T cells from PBMCs or TILs. $CD8^+$ T cells (1–4) or anti-CD3/CD28-activated $CD8^+$ T cells (5–8) from both PBMCs and TILs were cultured in conditioned media derived from IFNγ-treated (2, 4, 6, 8) or untreated (1, 3, 5, 7) Eca109 cells for 4 d, and the population of apoptotic cells was detected by flow cytometric analysis, using Annexin V and Propidium Iodide as indicators. Columns, mean of three independent experiments. Bars, SD. $^*P < 0.05$ compared with $CD8^+$ T cells cultured in conditioned media derived from non-IFNγ-treated Eca109 cells.

of $CD8^+$ T cell proliferation locally by IDO expression in ESCC tumor cells may contribute to the high IDO expression correlated with low numbers of $CD8^+$ TILs both in the tumor epithelium and tumor stroma.

In addition, we presented the evidence that exposure to IDO-expressing Eca109-CM dramatically weakened the cytolytic function of $CD8^+$ T cells from both PBMCs and TILs against target cells, but this attenuation could be abrogated by the addition of the IDO inhibitor 1 MT. In line with our observations, Liu et al. very recently reported that in the experimental rat lung allograft model, IDO creates a local microenvironment that leads to not only reduction in the numbers of $CD8^+$ TILs, but also the loss of cytotoxic activity of the $CD8^+$ effector T cells toward target cells [22]. The impaired cytotoxic function seen in the IDO-treated $CD8^+$ T cells was accompanied by defects in the production of

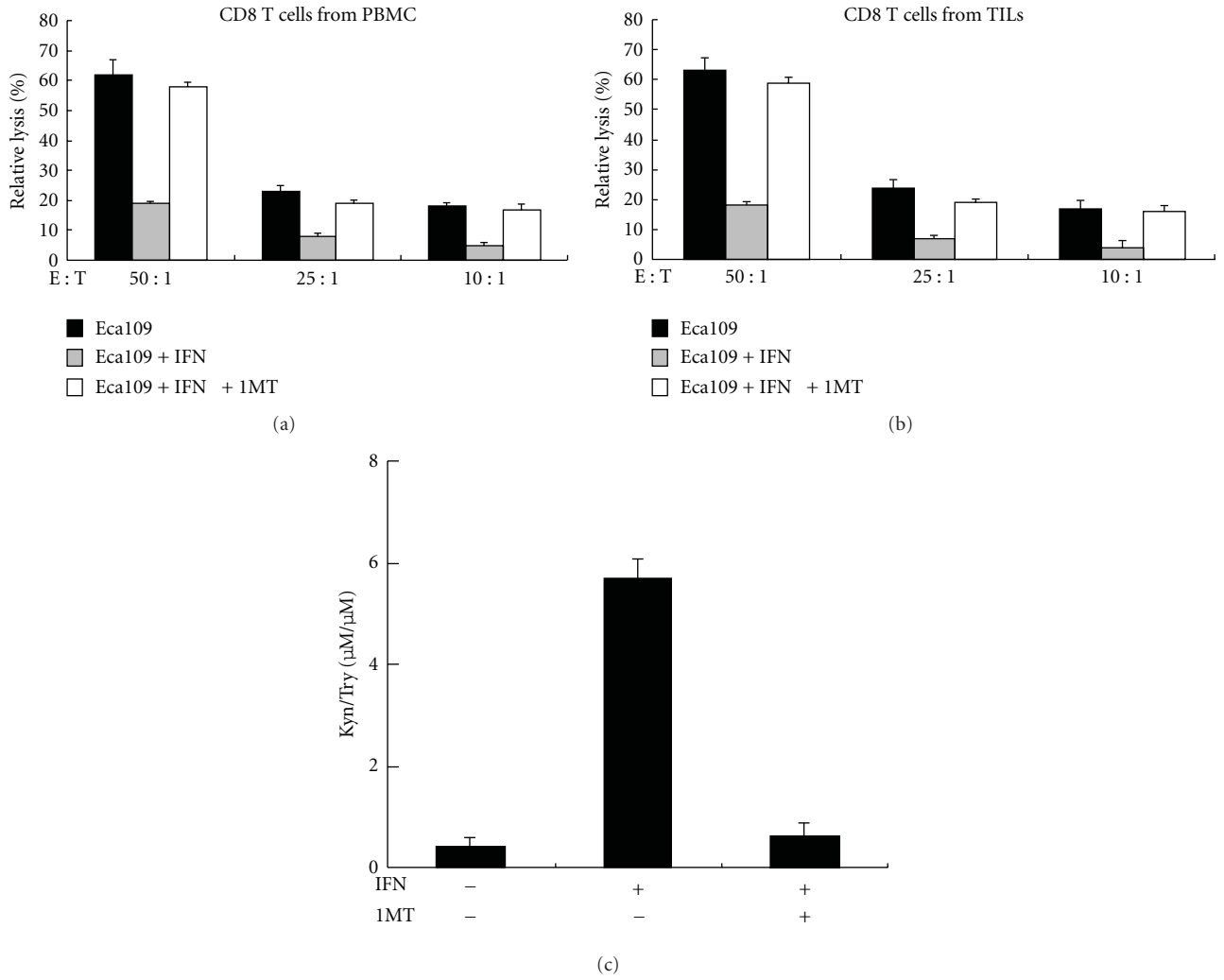

Figure 6: Cytolytic activity of $CD8^+$ T cells from both PBMCs and TILs was impaired by incubation with media created by IDO-positive Eca109 cells. Activated $CD8^+$ T cells from both PBMCs (a) and TILs (b) were cultured with CM from Eca109 cells treated with or without 50-U/mL IFNγ and/or 100-μM 1 MT for 24 hr, and cytolytic activity against Eca109 cells was evaluated using a standard LDH release assay. The E/T ratios are indicated. (c) Eca109 cells were treated with or without 50-U/mL IFNγ and/or 100-μM 1 MT for 24 hr. The Eca109 cell supernatants were analyzed for Kyn/Try production by HPLC. Columns, mean of three independent experiments. Bars, SD. $^*P < 0.05$ compared with $CD8^+$ T cells cultured in conditioned media derived from non-IFNγ-treated Eca109 cells.

granule cytotoxic proteins, including perforin and granzyme A and B. Moreover, IDO leads to an impaired bioenergetic condition in active $CD8^+$ T cells via selective inhibition of complex I in the mitochondrial electron transfer chain [22]. Our previous study also showed that exposure to the milieu created by IDO-positive nasopharyngeal cancer cells significantly impaired lymphocytes against target tumor cells [23]. These findings, together with our observations, suggest that IDO creates an immune suppression microenvironment not only by suppressing the proliferation of $CD8^+$ TILs but also by impairing the cytotoxic function of $CD8^+$ TILs. However, further studies are needed to elucidate the exact mechanisms of how IDO expressed by ESCC tumor cells reduces the cytotoxicity of $CD8^+$ T cells.

In conclusion, IDO expression in ESCC correlated with the reduced number of $CD8^+$ TILs, which is associated with disease progression and worse clinical outcome, may largely be due to the IDO-mediated proliferation arrest of $CD8^+$ TILs. Moreover, the $CD8^+$ T cells exposed to the milieu generated by IDO-expressing Eca109 cells lost their cytolytic function. We suggest that the effect of IDO expressed in ESCC cells on the proliferation and cytolytic function of $CD8^+$ TILs could contribute to the finding that patients with higher IDO expression have more aggressive disease progression and a shorter overall survival time. Although the precise role of tumoral IDO in human ESCC remains to be elucidated, our findings suggest that blocking IDO activity may provide a potential means of restoring the host antitumor immunity in the treatment of ESCC.

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Review Article

Asbestos Induces Reduction of Tumor Immunity

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Asbestos-related cancers such as malignant mesothelioma and lung cancer are an important issue in the world. There are many conflicts concerning economical considerations and medical evidence for these cancers and much confusion regarding details of the pathological mechanisms of asbestos-induced cancers. For example, there is uncertainty concerning the degree of danger of the iron-absent chrysotile compared with iron-containing crocidolite and amosite. However, regarding bad prognosis of mesothelioma, medical approaches to ensure the recognition of the biological effects of asbestos and the pathological mechanisms of asbestos-induced carcinogenesis, as well as clinical trials to detect the early stage of mesothelioma, should result in better preventions and the cure of these malignancies. We have been investigating the immunological effects of asbestos in relation to the reduction of tumor immunity. In this paper, cellular and molecular approaches to clarify the immunological effects of asbestos are described, and all the findings indicate that the reduction of tumor immunity is caused by asbestos exposure and involvement in asbestos-induced cancers. These investigations may not only allow the clear recognition of the biological effects of asbestos, but also present a novel procedure for early detection of previous asbestos exposure and the presence of mesothelioma as well as the chemoprevention of asbestos-related cancers.

1. Introduction

The fact that inhaled asbestos causes malignant mesothelioma and lung cancer is an enormous medical and social problem. Canada's decision to expand asbestos mining and export to developing countries in which asbestos has not been banned is unsettling [1]. People are sometimes influenced by economical forces even though they know many people have suffered from malignant diseases caused by these minerals, and their decisions appear to be made for financial reasons.

In Japan, the asbestos issue erupted in the summer of 2005 [2–4]. Residents were suddenly informed that asbestos, which was used in large amounts from the early 1950s up to the early 1990s in Japan with a maximum usage of approximately 352,000 tons in 1974, caused malignant mesothelioma (MM). Residents that lived near the asbestos handling manufacturer Kubota Corporation, in Amazasaki City, Hyogo Prefecture, developed MM. They had never worked in the asbestos-handling manufacture industry. In addition, medical information regarding MM induced anxiety in the Japanese people, since the prognosis is very poor, and there is no certain way to detect the cancer in the very early stage of the disease. Furthermore, people could not remember being exposed to asbestos 30 to 40 years ago. To reduce the anxieties of the Japanese people, epidemiological analyses regarding the Amagasaki area proceeded, and clinical and basic research was conducted on the biological effects of asbestos and early detection of mesothelioma. It is in this context that the authors

became involved in the project "Comprehensive Approach on Asbestos-Related Diseases", supported by the "Special Coordination Funds for Promoting Science and Technology" (Dr. Takemi Otsuki, Department of Hygiene, Kawasaki Medical School, Kurashiki, Japan) from 2006 to 2010. In this project, a case and clinical specimen registration system was established. A feasibility clinical trial was established and involved a combined trimodality therapy using anticancer chemotherapy with cisplatin and pemetrexed, following by extrapleural pneumonectomy and postoperative radiation therapy for early-stage mesothelioma patients [5, 6]. Furthermore, early detection procedures were developed using serum or pleural effusions to measure soluble mesothelin-related peptide (SMRP) and other markers such as osteopontin, vascular endothelial growth factor (VEGF) and angiopoietin-1 [7–9], as well as procedures for detection of circulating mesothelioma cells and circulating epithelial cells using peripheral blood [10, 11].

For the basic research, the project "Comprehensive Approach on Asbestos-Related Diseases" included three subgroups: (1) analyses of cellular and molecular characteristics using mesothelioma cell lines, (2) investigation of asbestos-induced carcinogenesis using an animal model, and (3) study of the immunological effects of silica/asbestos.

The first subgroup explored novel tumor suppressor gene(s) in mesothelioma cells and found that the serine/threonine-protein kinase (LATS2) gene is inactivated in approximately one-third of mesothelioma cell lines and is a candidate for a novel tumor suppressor in MM [12]. In addition, they found the possibility that the Yes-associated protein (YAP) involved the NF2/Merlin-hippo signaling pathway and that LATS2 may constitutively dephosphorylate and act as an oncogene to bind with the TEAD transcription factor to enhance the cell cycle and resistance to apoptosis [13]. In addition, mesothelioma-specific epigenetic profiles were identified for differential diagnosis with lung adenomatous cancers [14].

The second subgroup confirmed the importance of iron in asbestos-induced carcinogenesis. Findings showed that not only iron-containing crocidolite and amosite, but also chrysotile asbestos caused mesothelioma when these materials were injected into the peritoneal region of a rat. Even individual rats having mesothelioma caused by the injection of iron-absent chrysotile showed numerous depositions of iron in the spleen, liver, and kidney. In addition, adding nitrilotriacetate (NTA) to chrysotile-injected rats induced the acceleration of mesothelioma formation, suggesting the critical participation of iron for asbestos-induced carcinogenesis even for chrysotile. Although the detailed mechanisms of this phenomenon are now being explored, the binding ability of chrysotile to hemoglobin and other proteins and the induced hemolysis is a concern [15–18]. Moreover, the importance of a homozygous deletion of CDKN2A/2B was found in rat mesothelioma with the suggestion that this deletion seems to be fundamental for the development of mesothelioma, since these genes are also known to be homozygously deleted in human mesothelioma [19].

We have performed the third subtheme concerning the "immunological effects of silica/asbestos". In this paper, we introduce our findings and considerations regarding involvement of reduced tumor immunity caused by asbestos exposure to immunocompetent cells as the basic condition in asbestos-exposed people who may develop MM.

2. Immunological Effects of Asbestos

Asbestos comprises a set of six naturally occurring silicate minerals (chrysotile as Serpentine and crocidolite, amosite, actinolite, anthophyllite, and tremolite as Amphibole) exploited commercially for their desirable physical properties. They all have in common their asbestiform structure, possessing long (having more than 1 : 3 aspect ratio, usually approximately 1 : 20) and thin fibrous crystals [20, 21]. Silica (SiO_2) certainly affects the human immune system, because people exposed to silica not only suffer from respiratory disorders known as silicosis, but also experience complications with autoimmune disorders such as rheumatoid arthritis (known as Caplan's syndrome), systemic sclerosis, systemic lupus erythematosus, and antineutrophil cytoplasmic antibody- (ANCA-) related vasculitis/nephritis [22–27]. We have, therefore, been exploring the mechanisms involved in silica-induced dysregulation of autoimmunity using case peripheral blood specimens. We had found that there is dysregulated expression of the CD95/Fas molecule, which is very important for the survival of self-recognizing T cell clones. Additionally, analyses of Fas and Fas-related molecules in silicosis patients suggested that there are two populations of T cells: one is the long-term surviving populations probably including self-recognizing clones, and the other is a population repeating apoptosis caused by silica and recruiting from the bone marrow [28, 29]. In addition, our recent studies regarding CD4+25+ and forkhead box P3 (FoxP3)+ regulatory T cells (Treg) suggested that (1) silica activates both responder T cells (Tresp) and Treg, (2) Tresp chronically-activated by silica becomes CD4+25+ (and programmed cell death-1 (PD-1) + as an activated cell marker) expressers, (3) Treg activated by silica express higher CD95/Fas and are sensitive to Fas-mediated apoptosis, and (4) after the ongoing progression of these events, the composition of the peripheral CD4+25+ fraction in silicosis patients changes to reflect a loss of Treg and a gain of activated Tresp, and this reduction of Treg function results in activation of autoimmunity in silicosis patients [30–32].

Since silica influences the human immune system, its mineral silicate, an asbestos, may also have an effect. As we considered silica's immunological effects from the complications of silicosis and autoimmune diseases, the most important complication of asbestos-exposed people is the occurrence of malignant disease such as MM and lung cancer. In addition, some epidemiological studies suggested a relationship between asbestos exposure and other cancers of the gastrointestinal tract, larynx, kidney, liver, pancreas, ovary, and hematopoietic systems [33–35]. Thus, if asbestos affects the immune system, a reduction of tumor immunity may result and then make people exposed to asbestos

sensitive to the development of malignancies. Of course, asbestos itself possesses carcinogenic activities. As shown in Figure 1, asbestos fibers having iron (or even chrysotile as mentioned above) produce reactive oxygen/nitrogen species (ROS/RNS) causing DNA damage to nearby cells, and fibers are sometimes directly inserted into the cells and injure chromosomes, while retained fibers may adsorb other carcinogens on their surface (known as an asbestos body) [15, 16, 18, 36, 37]. As a result, specific DNA alterations may result, such as inactivation (mostly homozygous deletion) of $p16^{INK4a}/p14^{ARF}$, *NF2/Merlin,* and *LATS2*, and the activation of *YAP* as mentioned above [12, 13]. However, it is difficult to explain why the development of mesothelioma requires 30 to 40 years, and how asbestos-exposed people possess sensitive features for other cancers.

We have been considering that asbestos may affect immunocompetent cells such as CD4+ Tresp, Treg, Th17 T cells, CD8+ cytotoxic T cells (CTL), monocyte-macrophage, natural killer (NK) cells, natural killer T (NKT) cells, and dendritic cells (DC). Firstly, to observe the effects of low-dose and continuous exposure to asbestos (we initially chose chrysotile because this is the most frequently used asbestos in Japan, and our investigations suggested it was not carcinogenic), we employed a human adult leukemia/lymphoma virus-1 (HTLV-1) immortalized polyclonal T cell line, MT-2 [38, 39]. In the next part of this paper, analysis of asbestos exposure to the MT-2 cell line is documented.

3. Transient and Continuous Exposure to Asbestos on a Human T Cell Line

Initially, the cellular alteration of MT-2 cells exposed to transient and high-dose chrysotile was observed to compare various published investigations showing the ability of asbestos exposure to induce ROS production and mitochondrial-pathway-dependent apoptosis in normal alveolar epithelial cells and mesothelial cells, which are the target cells of asbestos-induced carcinogenesis. As shown on the left side of Figure 2, transient and relatively high-dose exposure (25–50 μg/mL, not likely to comprise adhesive cells such as alveolar epithelial or mesothelial cells, since we are using suspended cells, and thus, μg/mL was used instead of μg/cm^2) caused production of ROS as measured by production of O_2^- using flow cytometry, phosphorylation of proapoptotic molecules in the mitogen-activated protein kinase (MAPK) pathway such as p38 and c-Jun N-terminal kinase (JNK), release of cytochrome-c from mitochondria to the cytosol, BAX overexpression, cleavage of caspase-9 and -3, and thereafter the appearance of apoptosis [40]. These findings resembled the effects of asbestos on alveolar epithelia and mesothelial cells [41–44].

We then conducted a trial to establish a low-dose and continuous exposure cell line model by adding 5 or 10 μg/mL of chrysotile (doses which cause apoptosis in less than half of cells exposed transiently) to the MT-2 cell culture. After more than eight months exposure with monthly monitoring for the occurrence of apoptosis, and when these cells were re-exposed to fibers one week after being released from continuously exposed chrysotile, an MT-2 subline which showed resistance to chrysotile-induced apoptosis had been established. As shown on the right side of Figure 2, the continuously exposed subline of MT-2 showed activation of Src-family kinase, increased expression and production of interleukin (IL)-10, phosphorylation of signal transducer and activator of transcription 3 (STAT3) with overexpression of BCL-2 (located downstream of STAT3) [45, 46]. In addition, transforming growth factor (TGF)-β was upregulated [47, 48]. Actually, we had established three independent continuously exposed sublines to chrysotile B and three other sublines exposed to chrysotile A. The altered gene expression of these six continuously exposed sublines in comparison with the original MT-2 cell line was very similar, and the cellular and molecular characteristics of these cell lines in regard to tumor immunity with the *ex vivo* chrysotile exposure model using freshly isolated peripheral blood CD4+ T cells derived from healthy donors was investigated and confirmed using peripheral blood specimens derived from asbestos-exposed patients such as patients with pleural plaque (PP) or MM.

Chemokine receptor, CXC chemokine receptor (CXCR)3, expression and relation with interferon (IFN)-γ.

Using the above-mentioned MT-2 original cell line and the continuously exposed chrysotile sublines, molecules related to tumor immunity were investigated. For example, CXCR3 expression was a focus of investigations, since CXCR3 downregulation in sublines was detected in comparison with the original line using cDNA microarray analysis. It is known that CXCR3 expression and IFN-γ production are induced by T-cell activation and lead to the enhancement of antitumor immune function [49, 50].

From findings using the MT-2 cell line model, as shown in Figure 3(a), all six continuously exposed sublines showed reduced CXCR3 expression on their surface and mRNA expression levels with reduced production and expression of IFN-γ. Production of the Th1-type CXCR3 ligand CXCL10/IP10 was also significantly reduced in all six continuously exposed sublines when compared with the original line. In addition, another Th1-type chemokine, CCL4/MIP-1β mRNA, was also expressed at low levels in all six sublines compared with the MT-2 original line as previously reported. However, CCR5, the Th1-type receptor for CCL4/MIP-1β, was not reduced significantly through mRNA expression in MT-2Rsts cells. These results indicated that continuous exposure of MT-2 original cells to asbestos altered the expression of Th1-related chemokines (CXCL10/IP10 and CCL4/MIP-1β) and chemokine receptors (CXCR3) [51].

Thereafter, we tried to determine whether freshly isolated human peripheral CD4+ T cells show a similar alteration *ex vivo* when proliferation is maintained by IL-2-containing medium in the presence of chrysotile as shown in Figure 3(b). After 40 days of coculture supplemented with IL-2 in the presence or absence of chrysotile, cell surface CXCR3 expression decreased in a dose-dependent manner. Thus, we examined cell surface expression of CXCR3 and CCR5 in CD4+ T cells derived from six healthy donors, since both receptors are preferentially expressed in Th1/effector T cells. The expression of CXCR3 was significantly reduced

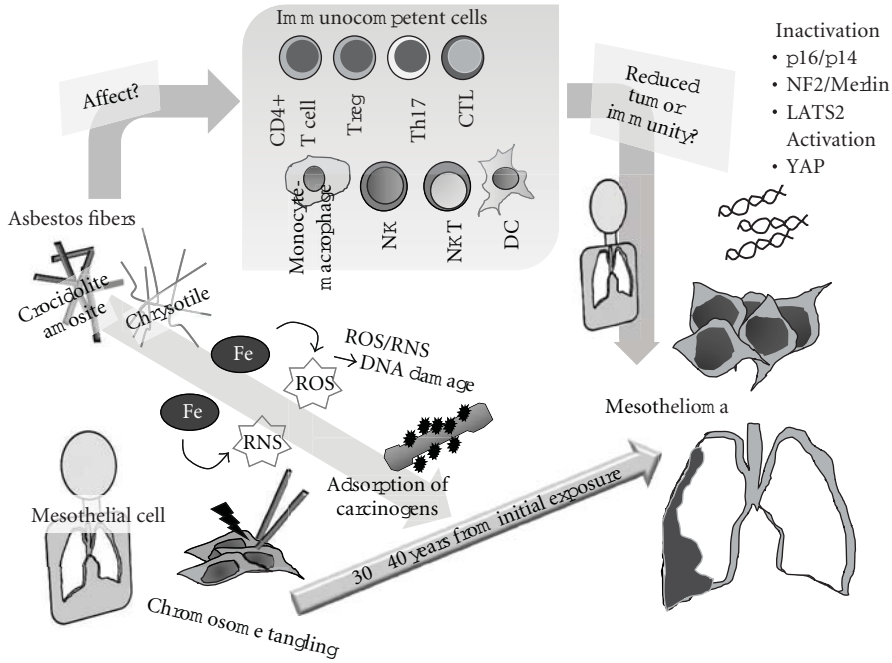

Figure 1: Schematic model showing mechanisms of asbestos-induced carcinogenesis and genomic/epigenetic changes found in mesothelioma cells and the relationship of the immunological effects of asbestos in regard to reduced tumor immunity.

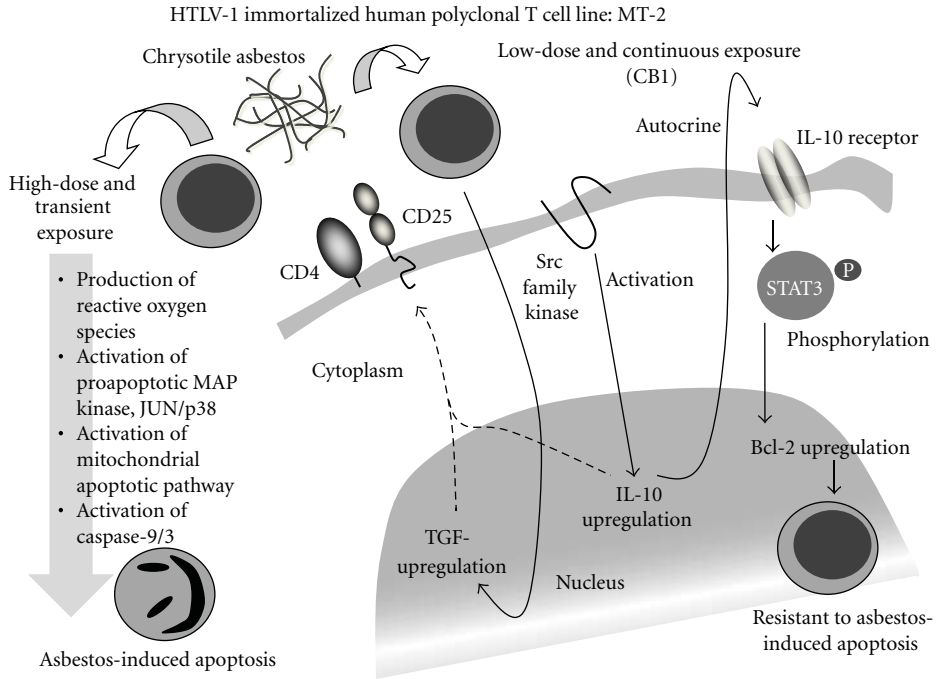

Figure 2: Summarized findings of cellular and molecular events caused by high-dose and transient exposure (left side) and lowdose and continuous exposure (CB1: one of the sublines established) (right side) to chrysotile asbestos using an HTLV-1 immortalized human polyclonal T cell line, MT-2.

following exposure to 10 μg/mL of chrysotile for 28 days although this difference seemed to depend on one case in which the expression decreased remarkably. Even if the culture conditions for the CD4+ T cells was limited to a period of around four weeks, four of the six healthy donors showed a decrease of CXCR3 expression to various degrees, and it might be concluded that asbestos exposure potentiates reduction of CXCR3 expression in CD4+ T cells. On the other hand, the expression of CCR5 varied among all healthy donors, and there were no significant changes after

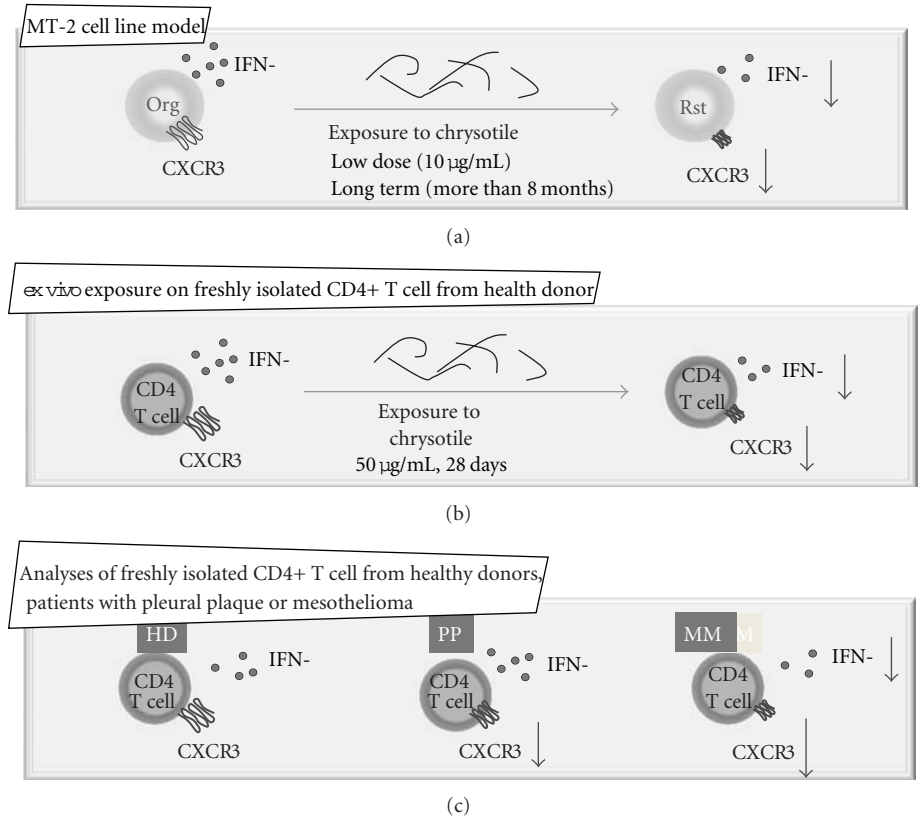

Figure 3: Schematic representation of asbestos-induced reduction of expression of a chemokine receptor, CXCR3, and expression and production of IFN-γ using the MT-2 cell line model (Org; MT-2 original cell line, and Rst: sublines exposed continuously to a low-dose of chrysotile), an *ex vivo* exposure model using freshly isolated CD4+ T cells from healthy donors (HD), as well as analyses of freshly isolated CD4+ T cells from healthy donors and patients with pleural plaque (PP) and malignant mesothelioma (MM).

seven and 28 days of coculture with chrysotile, as shown previously by the cell line model. These results indicated that CXCR3 expression might be specifically reduced by asbestos exposure. In addition, these experiments revealed decreased IFN-γ expression and production when CD4+ T cells from healthy donors were cultured with chrysotile for 28 days [52].

Finally, analyses of changes in surface CXCR3 expression on freshly isolated CD4+ T cells from asbestos-exposed patients such as PP or MM were compared with those from health donors. In addition, IFN-γ expression of CD4+ T cells from these patients and healthy donors was measured with stimulation using anti-CD3/CD28 antibodies with IL-2. As summarized in Figure 3(c), CXCR3 expression was reduced in CD4+ T cells from asbestos patients. A comparison of PP and MM patients showed that the expression level of CXCR3 on CD4+ T cells from MM was decreased although the difference was not statistically significant. Moreover, IFN-γ expression was only reduced in stimulated CD4+ T cells from MM patients, not in those from PP patients [52].

With the findings that CD4+CXCR3+ T cells in lymphocytes from MMs showed a tendency for an inverse correlation with CXCR3's ligand, CXCL10/IP10 in plasma, our results indicate a reduction of tumor immune function in asbestos-exposed patients and suggest that CXCR3, IFN-γ, and CXCL10/IP10 may be candidates to detect and monitor disease status.

4. Alteration of NK Cells and Others

As shown in Figure 4, the effects of asbestos on other immunocompetent cells such as Treg, CD8+ CTL, and NK cells were investigated. As mentioned above with the MT-2 cell line model, sublines continuously exposed to chrysotile showed overproduction of IL-10 and TGF-β. It is well known that these cytokines are a typical soluble factor produced from Treg to function with a suppressive effect on activated responder T cells. On the other hand, it is also reported that MT-2 cells possess a Treg function, since cells express CD4 and CD25 with nuclear expression of FoxP3. Taken together, continuous exposure to chrysotile produces a stronger Treg function, at least with the capacity to produce soluble functional factors (i.e., IL-10 and TGF-β) [47, 48]. At present, we have been studying alteration of Treg function using the MT-2 cell line model, and preliminary findings indicate asbestos may enhance Treg function.

In regard to tumor immunity, CD8+ CTL and NK cells are very important players, since they directly kill tumor cells even when individually restricted with major histocompatibility complexes. Investigations have just started with

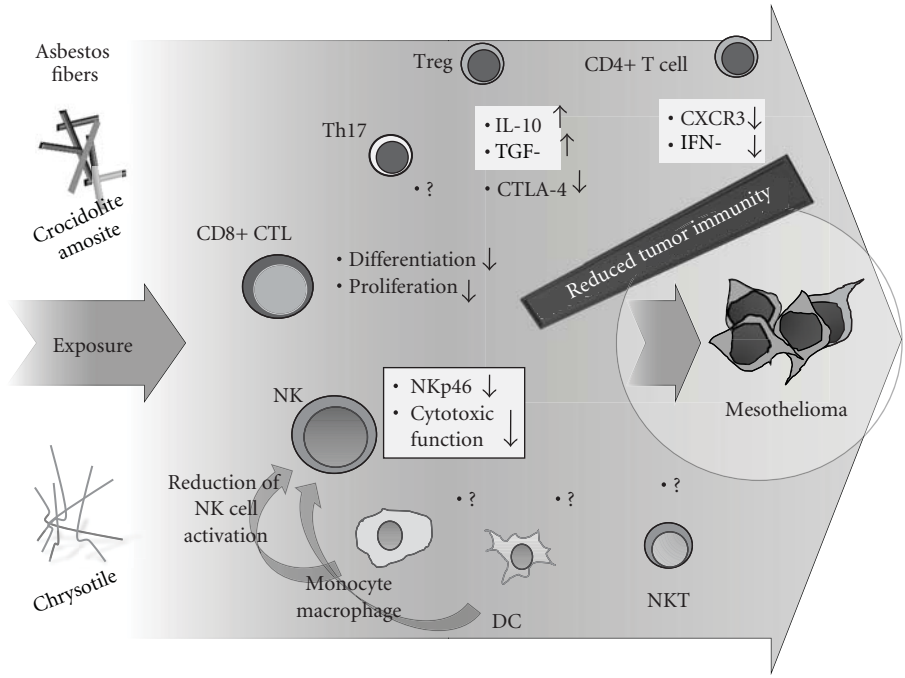

Figure 4: Schematic representation of findings showing asbestos-induced reduction of tumor immunity on CD4+ T cells, CD4+25+FoxP3+ regulatory T cells (Treg), T helper (Th)17, CD8+ cytotoxic T cells (CTL), natural killer (NK) cells, monocyte-macrophage, dendritic cells (DC), and natural killer T cells (NKT).

CD8+ CTL, but in *ex vivo* experimental conditions designed to produce CD8+ CTL proliferation and differentiation using freshly isolated peripheral blood mononuclear cells from healthy donors, the addition of asbestos seems to result in reduced proliferation and differentiation of CTL. Although detailed analyses concerning the roles of cytokines surrounding CTL differentiation are being performed, our ongoing studies suggest that asbestos reduces CTL activities.

Regarding NK cells, cellular and molecular analyses have been conducted using a human NK cell line, YT-A1, exposed continuously to asbestos in an *ex vivo* exposure model using freshly isolated NK cells from health donors, as well as asbestos-exposed patients such as PP and MM.

Focusing on the NK cell-activating receptors, including NKG2D (also known as KLRK1 (killer cell lectin-like receptor subfamily K, member 1), klr and CD314, binding to a diverse family of ligands that include MHC class I chain-related A and B proteins and UL-16 binding proteins, where ligand-receptor interactions can result in the activation of NK and T cells), 2B4 (also known as NAIL; Nmrk; NKR2B4; SLAMF4 and CD244, mediate nonmajor histocompatibility complex (MHC) restricted killing), and NKp46 (also known as NCR1 (natural cytotoxicity triggering receptor 1), LY94 and CD335, constituting a natural cytotoxic receptor family with NKp44 and NKp30, and being important in killing tumor cells and dendritic cells), the YT-A1 human NK cell line exposed continuously to chrysotile asbestos reveal-ed reduced expression of NKG2D and 2B4 [53]. The reduced phosphorylation of extracellular signal-regulated kinase (ERK) and subsequent reduction of degranulation of perforin and granzyme B resulting from reduced cytotoxicity were observed in this cell line model [54]. Similar to the cell line model, the *ex vivo* exposure model using freshly isolated NK cells from healthy donors revealed a reduction of NKp46 expression. Furthermore, freshly isolated NK cells from MM patients showed reduced killing function compared with those from healthy donors and revealed a lower expression of NKp46 [53]. Moreover, the expression level of NKp46, but not NKG2D or 2B4, and the cytotoxic activity of individual freshly isolated NK cells from health donors and MM patients clearly showed a reverse correlation, indicating that the target molecule of asbestos-induced dysfunction of NK cells is NKp46 [54]. Although further analyses are required regarding the interaction between asbestos-exposed NK cells and other immunocompetent cells such as dendritic cells, monocytes, and macrophages, molecular mechanisms to reduce NKp46 expression and other aspects need to be explored, and surface NKp46 expression levels may be the candidate to monitor the level of tumor immunity in asbestos-exposed patients [55].

Further investigations are needed to examine the effects of asbestos exposure on other types of immunocompetent cells such as Th17 dendritic cells, NKT, and the monocyte-macrophage lineage, and to investigate why asbestos seems to reduce tumor immunity in the total network of the immunological surveillance system.

In addition, although we have mainly analyzed the effects of chrysotile asbestos on the human immune system,

differences and similarities between the different types of fibers should also be investigated.

5. Conclusion

We have been investigating the effects of asbestos exposure on the human immune system in regard to tumor immunity and found that people exposed to asbestos possess reduced tumor immunity, making them sensitive to cancer development. Although these studies may contribute to the clear recognition of the biological effects of asbestos, the variety of alterations in immunocompetent cells may be the factor that allows detection of previous asbestos exposure and the occurrence of cancer in people that live or have lived near asbestos-handling manufacturers. Furthermore, to recover tumor immunity using physiologically active substances in foods or derived from plants may be an effective method for the chemical prevention of asbestos-induced cancers.

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Research Article

Dendritic Cell-Based Vaccines Positively Impact Natural Killer and Regulatory T Cells in Hepatocellular Carcinoma Patients

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Immunotherapy of cancer must promote antitumor effector cells for tumor eradication as well as counteract immunoregulatory mechanisms which inhibit effectors. Immunologic therapies of cancer are showing promise, including dendritic cell-(DC-) based strategies. DC are highly malleable antigen-presenting cells which can promote potent antitumor immunity as well as tolerance, depending on the environmental signals received. Previously, we tested a peptide-pulsed DC vaccine to promote Alpha-fetoprotein (AFP-) specific anti-tumor immunity in patients with hepatocellular carcinoma (HCC), and reported on the $CD8^+$ T cell responses induced by this vaccine and the clinical trial results. Here, we show that the peptide-loaded DC enhanced NK cell activation and decreased regulatory T cells (Treg) frequencies in vaccinated HCC patients. We also extend these data by testing several forms of DC vaccines *in vitro* to determine the impact of antigen loading and maturation signals on both NK cells and Treg from healthy donors and HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide [1]. It often follows cirrhosis caused by viral or alcoholic hepatitis. Prognosis remains very poor, and treatment options are few [1]. Curative surgery and liver transplantation are only available to a small minority of early-stage HCC patients. Other common therapies (including ablative therapies and Sorafenib) are largely palliative. Treatment is complicated by preexisting cirrhosis, as chemotherapy or resection may not be options in a patient with poor liver reserves.

Alpha-fetoprotein (AFP) is an oncofetal antigen that is expressed by more than half of HCC tumors and detectable at elevated levels in the blood and tumor microenvironment in these HCC patients [2]. AFP serves as the most common serum biomarker for HCC and, as it is from undetectable to 10 ng/mL in healthy adults [3], has also been identified as a specific tumor-associated antigen for HCC immunotherapy [4]. We and others have investigated AFP as a tumor rejection antigen for immunotherapy of HCC [5–13].

Dendritic cell (DC) vaccines are promising vehicles for activating antitumor specific T cells and NK cells for tumor immunotherapy. They are immunologic sentinels which can induce antigen-specific immunity or tolerance [14, 15]. DC can be activated or matured with cytokines and toll-like receptor (TLR) agonists such as interferon gamma (IFNγ) or lipopolysaccharide (LPS) [16–19]. Antigen loading of DC can be achieved in a number of ways, including peptide pulsing, whole protein loading, and genetic engineering via viral transduction.

While DC are critical to induction of immunity, other immune cells are important as effectors and regulators in cancer immunity. $CD56^+CD16^{+/-}$ natural killer (NK) cells are the effectors of the innate immune system that are able to directly kill tumor or virally infected cells with reduced levels of MHC class I molecules or that overexpress stress-induced activating cell surface molecules (e.g., MICA/B recognition via NKG2D), and that otherwise may escape immune detection. Hepatic lymphocytes are enriched (up to 30%) in NK cells that may play a role in antitumor defense [20].

Table 1: HCC patient demographics.

Pt.	Dose DC	Risk Factor	Stage	Previous treatments[1]	Pre-AFP (ng/mL)	Post-AFP (ng/mL)	Response[2]	PFS[3] (mo)	OS[4] (mo)
A1	1×10^6	?	IVb	Chemoembo, CDDP, Adriamycin, 5-FU, Xeloda, Thalidomide	2.811	2.748 (+28)	PD	0	4
A2	1×10^6	HBV	IVa	Chemoembo	4.740	5.770 (d + 28)	PD	0	20
A3[5]	1×10^6	EtOH	IVa	RFA	3.080	(no DC)	(no DC)	0	2
A4[6]	1×10^6	HCV	IVb	—	10,800	10.650	(1 vaccine)	0	—
B2	1×10^6	?	IVa	Surgery	5.100	7.650 (d + 35)	PD	0	4
B3[7]	5×10^6	HCV	IVa	Chemoembo RFA	102	65 (d + 28)	NE	0	35
B5	5×10^6	HBV	IVa	Chemoembo, CarboTaxol, Xeloda	1.630	2.515 (d + 112)	PD	0	3+
B8	5×10^6	HCV	IVb	Chemoembo	96.7	134	PD	0	5+

[1]Previous treatments received (chemoembo, chemoembolization; CDDP, cis-platin; 5-FU, 5-flouro-uracil; Xeloda, capecitabine; RFA, radiofrequency ablation; carbo, carboplatin; XRT, radiation therapy).
[2]PD: progressive disease, NE: no evidence of disease.
[3]PFS: progression free survival.
[4]OS: overall survival.
[5]No DC: no DC vaccines could be generated which passed clinical protocol release criteria.
[6]1 vaccine: patient progressed early and did not receive the 3 DC vaccinations.
[7]NE: patient B3 responded to chemoembolization and RFA and was vaccinated shortly thereafter, and had 35 mo. OS.

Murine models support a role for NK cell effectors in liver tumor responses. In mice, antitumor effects mediated by NK cells were IFN-γ dependent [21]. The $CD4^+CD25^{hi}FOXP3^+$ T regulatory (Treg) cell has more recently been recognized as an important target in immunotherapy because of its role in inhibiting the immune response. Patients with HCC have been shown to have defects in NK cell function [22] and high intratumoral [23] and circulating levels of Treg [24], all of which may impact the progression of this disease.

We previously tested an AFP peptide-pulsed DC vaccine in a phase I clinical trial. The vaccine was found to be safe and immunogenic in late-stage HCC patients [25–27]. We detected type I immunity induced to the 4 immunizing HLA-A*0201-restricted AFP-derived peptides in the majority of patients by IFN-γ ELISPOT and MHC class I tetramer assays.

It has been demonstrated that DC and NK cells are capable of interacting with and activating each other [28–30]. We have found that recombinant adenovirus (AdV)-transduced DC (AdV/DC), unlike immature DC, are capable of functionally activating NK cells [17]. There are also circumstances in which DC can promote Treg expansion. In this study, we examined the *in vivo* effects of AFP peptide-pulsed DC on NK cell activation and Treg frequencies and phenotypes in peripheral blood mononuclear cells (PBMC) of HCC patients and described evidence for both NK cell activation and decreased frequencies of $FOXP3^+$ Treg cells. We then compared several clinically relevant DC preparations for effects on NK cells and Treg *in vitro* and find differences in the DC groups and between HCC patients and healthy donors (HD). We show that AdV/DC, with (pmAdV/DC) or without maturation, are most successful at inducing NK cell activation and Treg depletion. The results have relevance for the design DC-based vaccines in patients with HCC.

2. Materials and Methods

2.1. Patient and Healthy Donor Cells. PBMC were obtained from healthy volunteers (HD) and from HCC patients enrolled in a peptide-pulsed DC vaccine (UCLA IRB #00-01-026, IND BB9395; UPCI #04-001 and #04-111; informed consent was obtained from all patients and donors). The clinical trial was previously published in detail [26] which included immunologic monitoring of vaccine responses from banked PBMC. Limited patient data is listed in Table 1. PBMC were isolated using a Ficoll gradient and either tested fresh (some HD) or were cryopreserved (some HD and all HCC patient cells) in RPMI1640/20% human AB serum/10% DMSO for later testing.

2.2. Flow Cytometry. Cells were stained according to manufacturer recommendations, fixed in 0.5% paraformaldehyde, and analyzed on an CyAn high-speed analyzer (Dako, Carpinteria, Calif) (UPCI Flow Cytometry Facility) and the Summit v4.3 software within four days. NK cell phenotype was investigated using: CD8 PE, CD16 ECD, CD3 APC (Beckman Coulter), granzyme B FITC, CD25 PE-Cy7, CD56 PE-Cy5, and CD69 APC-Cy7 (BD Pharmingen). Treg were investigated using: CD4 FITC, FOXP3 PE, and CD25 APC (eBioscience) and reported as either the FOXP3 positive percentage or the MFI of FOXP3 expression in the $CD4^+CD25^+$ cells.

2.3. Cell Isolation, DC Growth, and Vaccine Models (See Figure 1). $CD56^+$ NK cells and $CD4^+$ T cells were isolated from PBMC (Miltenyi Biotech) according to the manufacturer's directions (CD56 beads, NK isolation kit, $CD4^+$ T cell isolation kit). Change in MFI was considered "positive" if the increase was ≥25% of the baseline MFI. Percent positivity was considered positive if ≥5% greater than baseline.

Monocytes were isolated from PBMC using adherence to T75 flasks (Costar). They were cultured for 6-7 days in RPM1640/5% human AB serum/PennStrep medium with 500 U/mL IL-4 and 800 U/mL GM-CSF (Schering-Plough, Kenilworth, NJ; Amgen, Thousand Oakes, Calif) to promote differentiation to myeloid DC.

After culture, DC were harvested, counted (Trypan Blue Stain; BioWhittaker, Walkersville, MD), and cultured as described below. DC were subsequently cocultured with NK cells or T cells isolated from the autologous donor and incubated 24 hr (NK and CD4) to 5 days (CD4) at ratios of 1 DC to 1–10 NK or T cells. After the coculture, cells were harvested, supernatant was collected and stored at −80°C, and cells were analyzed by flow cytometry as described above.

For peptide-pulsed DC (pep/DC), DC were pulsed with 1 or 2 specific AFP peptides (AFP_{158} FMNKFIYEI and AFP_{542} GVALQTMKQ; synthesized at the University Pittsburgh Peptide Synthesis Facility) at 10 μg/mL for 2 hr at 37°C, then washed in medium before further culture. Similarly, for protein-pulsed DC (prot/DC), DC were loaded with cord blood-derived hAFP protein (CalBiochem) at 10 μg/mL for 2 hr at 37°C, then washed.

For AdV-transduced DC (AdV/DC), DC were transduced for 2 hr at 37°C in serum-free media (IMDM) at MOI = 1,000 with an AdV encoding full length AFP protein (AdVhAFP) [5] and then washed in medium before further culture. In the case of "prematured" AdV-transduced DC (pmAdV/DC), DC were first matured 24 hr with 250 ng/mL LPS (Sigma) and 1000 U/mL IFN-γ (Pepro Tech), after which they were washed and transduced with AdVhAFP as described above.

2.4. Luminex. Cell-free supernatants were collected from cultures and frozen at −80°C. They were subsequently thawed and simultaneously analyzed with the multiplex Luminex assay (Invitrogen) per manufacturer's protocol in a BioRad reader (UPCI Immunologic Monitoring Laboratory). The following analytes were tested: GM-CSF, IFN-γ, IP-10, MCP-1, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, and IL-10 in a kit pretested for any potential crossreactivity by the manufacturer. Controls included the included standard curve and multiplex QC standards (R&D Systems).

2.5. Statistical Analysis. One-tail *t*-test analyses were used to estimate statistical significance of differences obtained; *P* values ≤0.05 were considered to be statistically significant.

3. Results

Based on our previous study (17), we hypothesized that HCC patients vaccinated with immature DC pulsed with AFP peptides (pep/DC) would not impact activation of circulating NK cells. We assessed this by evaluating upregulation of CD69 or CD25 activation markers on $CD56^{hi}/CD16^-$ and/or $CD56^{lo}/CD16^+$ NK cell subsets. We also wished to determine whether Treg frequency (as determined by a change in FOXP3-expressing $CD4^+CD25^{hi}$ T cells) was modulated by vaccination, which might also include changes in CTLA-4 [31, 32] or CCR7 [33] expression on Treg.

3.1. HCC Patients Treated with AFP Peptide-Pulsed DC Exhibit Elevated Levels of NK Cell Activation. We tested banked PBMC samples from five HCC patients, isolated at different time points during vaccination with AFP pep/DC. Cells were stained immediately after thawing to assess phenotype by flow cytometry (see analysis strategy shown in Supplemental Figure 1 in Supplementary Material available online at doi:10.1155/2011/249281). Contrary to our hypothesis, both regulatory $CD56^{hi}CD16^-$ and cytotoxic $CD56^{lo}CD16^+$ NK cells demonstrated activation post-pep/DC vaccination, compared to baseline. Activation was determined by both an increase in population MFI (Figure 1) as well as percent positivity for CD25 or CD69 (Supplemental Figure 2). CD69 expression was increased in 4/5 patients for both $CD56^{lo}CD16^+$ and $CD56^{hi}CD16^-$ NK cells according to MFI and percent positive values (Figure 1 and Supplemental Figure 2). CD25 expression was also increased by percent positivity and MFI in 3/5 and 4/5 patients' $CD56^{lo}CD16^+$ NK cells (Figure 1). For $CD56^{hi}$ NK cells, CD25 was increased in 4/5 patients by MFI only. Patient B2 ($CD56^{lo}CD16^+CD69$ percentage and $CD56^{hi}CD16^-CD69$ MFI) and B8 ($CD56^{lo}CD16^+CD69$ and CD25 by both MFI and percentage) had a decrease in NK cell activation after first vaccination, with an increase after second immunization. The other patients displayed stronger NK cell activation. Of the two main NK cell subsets, $CD56^{lo}CD16^+$ cells showed the greater degree of activation. Patients A1, A4, and B5, with multiple time point samples available, had highest NK cell activation after the first round of vaccination, with subsequent time points showing less activation. Additionally, we tested these cells for the expression of NKG2D, an NK cell activating receptor; however only low levels of this molecule were detected on a small percentage of circulating NK cells (data not shown).

3.2. HCC Patients Treated with AFP Peptide-Pulsed DC Vaccines Display Decreased Frequencies of Circulating Treg Cells. To examine Treg cell frequencies, $CD3^+CD4^+$ T cells were gated on $CD25^{hi}$ or total $CD25^+$ and intracellularly stained for FOXP3. The Treg lymphocyte frequencies were then assessed by flow cytometry. FOXP3 expression in the $CD3^+CD4^+CD25^{hi}$ T cells showed a consistent change, decreasing overall in 4/5 of the patients tested, by both percent positivity and MFI (Figure 2). Similar to the NK cell activation measures, patient B2 also showed inferior Treg changes by either measure. Treg frequencies slightly increased in this patient after second and third immunizations. $CD3^+CD4^+CD25^{hi}$ cell surface CCR7 and CTLA-4 showed minor variation over time and were not considered informative in our data set (data not shown).

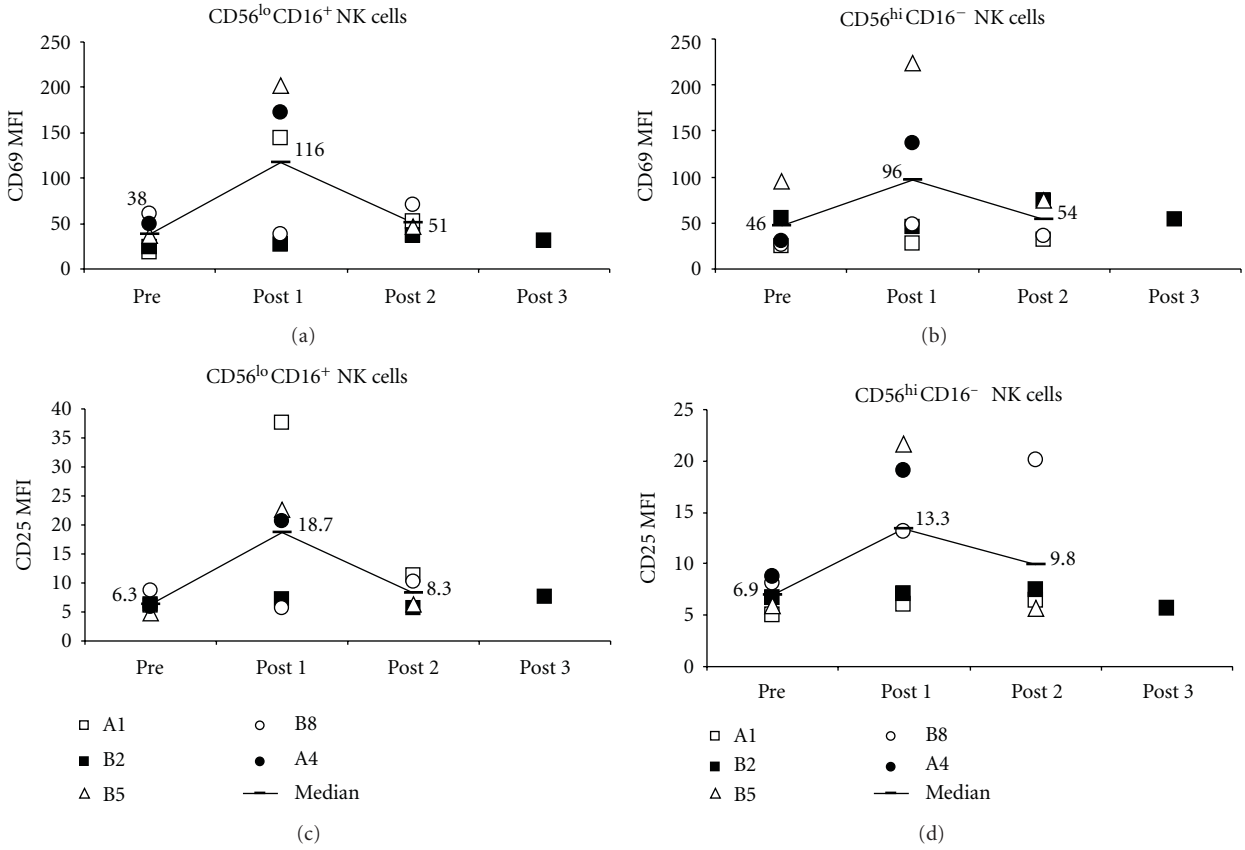

Figure 1: CD69 and CD25 expression on $CD56^{lo}CD16^{+}$ and $CD56^{hi}CD16^{-}$ NK cells. Phenotyping of NK cells from patients who received the AFP pep/DC vaccine, showing longitudinal changes. "Pre" denotes PBMC from time point 0. DC vaccines were delivered (after blood draws) on days 0, 14, and 28. "Post" denotes PBMC from postvaccine administration at time points available in the remaining batched PBMC. Patient A1 tested at days 35 and 56 (7 days and 28 days after the third vaccine); pt B2 at d28, 56, and 112; pt B5 at d14 and 28; pt B8 and d14 and 112, pt A4 at d14. CD69 and CD25 markers (by MFI) are shown for both NK cell subsets. Percent positivity is shown in Supplementary Figure 2. One-tail *t*-test *P* values are: $CD56^{lo}CD16^{+}$CD69 MFI: pre to post 1: 0.05; pre to post 2: 0.03; post 1 to post 2: 0.16; all other values higher and not significant (n.s.). $CD56^{hi}CD16^{-}$CD69 MFI: pre to post 1: 0.07, all other values higher and n.s. $CD56^{lo}CD16^{+}$CD25 MFI: pre to post 1: 0.05, pre to post 2: 0.11, post 1 to post 2: 0.12, all other values higher and n.s. $CD56^{lo}CD16^{-}$CD25 MFI: pre to post 1: 0.04, pre to post 2: 0.15, all other values higher and n.s.

3.3. Phenotypic Changes in DC with Di erent Antigen Loading Strategies. The AFP peptide-pulsed DC did not undergo a specific maturation step during vaccine preparation. Maturation cocktails can impact surface levels of MHC class I and II, costimulatory molecule levels, and cytokine production. We hypothesized that different DC antigen-loading strategies, some of which impact DC maturation, would result in unique phenotypic changes in the DC that would impact activation and frequencies of other immune cells (like NK cells and Treg) they interacted with. We previously tested AdV-mediated genetic engineering of DC to enable expression of full length antigens in DC [6, 34–37], and found that AdV transduction promotes partial maturation of DC and superior antigen-specific T cell responses. Since we have identified AFP as a tumor-associated T cell antigen for HCC [5, 10], we utilized the AFP antigen in multiple forms (AdVhAFP, AFP protein, AFP-derived peptide) for DC loading. We transduced HD DC with AdVhAFP and compared them to immature DC (iDC) and LPS/IFN-γ-matured DC (mDC). DC groups were then cocultured with autologous NK or $CD4^{+}$ T cells to determine the impact on these lymphocytes. We examined CD83, CCR7, CD86, and CD80 as markers of DC maturation. We found that all four markers were upregulated after AdV transduction as compared to iDC, but that their greatest upregulation was observed in mDC (data not shown, similar to [19, 37]). We also found that coculture with resting NK cells modestly improved the expression of these DC maturation markers in comparison to the level of modulation achieved by AdV transduction or LPS/IFN-γ treatments alone (data not shown, similar to [17]).

3.4. Changes in NK Cell Activation Levels and Treg Frequencies after Culture with DC. We hypothesized that NK cells would be activated (as measured by increased CD69 and CD25 expression) and that Treg frequencies might be reduced (decreased FOXP3 expression) after interactions with DC that were at least partially matured and that these trends would be observed after coculture with the AdV/DC.

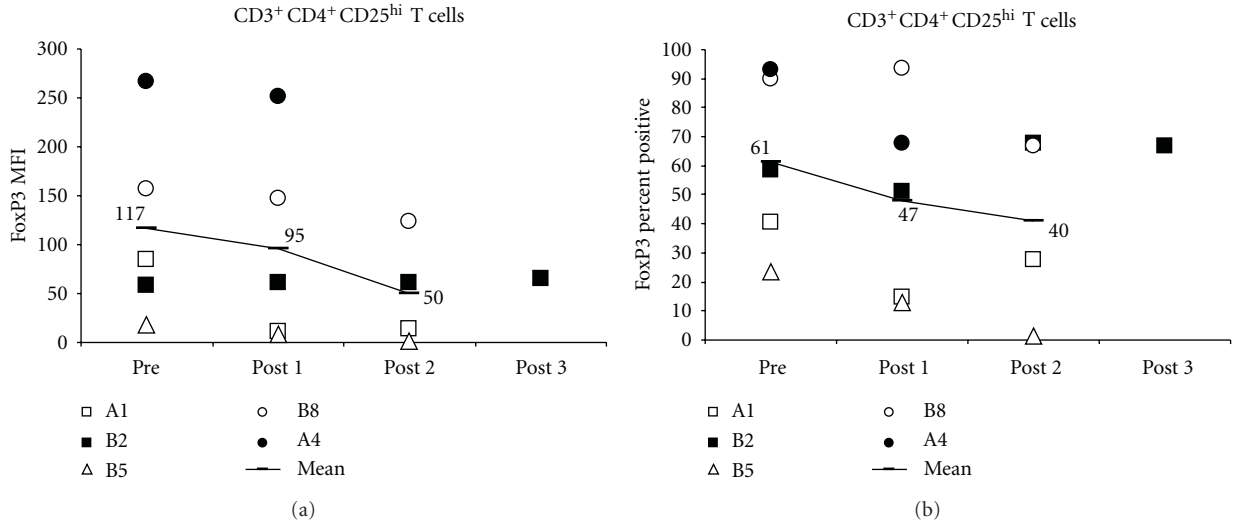

Figure 2: FOXP3 expression in $CD4^+CD3^+CD25^{hi}$ (Treg) cells. Phenotyping of Treg cells from patients who received the pep/DC vaccine, showing longitudinal changes. "Pre" denotes PBMC from time point 0. "Post" denotes PBMC from postvaccine administration at time points available. Patient A1 tested at days 35 and 56; pt B2 at d28, 56, and 112; pt B5 at d14 and 28; pt B8 and d14 and 112, pt A4 at d14. (a) FOXP3 assessed intracellularly in $CD3^+CD4^+CD25^{hi}$ cells, showing MFI, or (b) % positivity. One-tail *t*-test *P* values are: FOXP3 MFI: pre to post 1: 0.09, pre to post 2: 0.07, post 1 to post 2: 0.17; all other values higher and n.s. FOXP3 percent positive: pre to post 1: 0.03; pre to post 2: 0.10; all other values higher and n.s.

Monocyte-derived DC were antigen-loaded as described and cocultured with autologous NK or $CD4^+$ cells for 24 hr or 6 days, respectively. The cells were then harvested and assessed by FACS for phenotypic changes in specific subsets.

We first assessed different AFP antigen loading modalities (peptide, protein and AdV) in HD cells. Peptide pulsing and protein-loading do not include any maturation agents and have been observed not to alter DC phenotype *in vitro* while AdV provides a partial maturation signal from the viral transduction. While we recently investigated the ability of AdVLacZ-transduced DC to interact with NK cells in depth [17], the present study focused on AFP, which has been reported to have immune suppressive functions [38, 39]. In line with these publications, we have observed that AdVhAFP/DC express less transmembrane TNF than AdVLacZ/DC (L. Vujanovic and LH Butterfield, unpublished data, 2011). Here, we confirmed that NK cells cocultured with DC upregulate CD69 (increase in MFI values (Figure 3(a)) and percent positivity (not shown)). Activation at 48 hr was somewhat stronger than at 24 hr (not shown). Sufficient HD cells were available to also test "pre-matured" (first matured with IFN-γ and LPS) then AdV-transduced DC (pmAdVhAFP/DC), which we found to more potently activate antigen-specific $CD8^+$ T cells than AdV/DC alone [19]. Overall, DC transduced with AdV and/or pre-matured and AdV-transduced were slightly more potent NK cell activators than pep/DC or prot/DC (which were similar to each other).

We then tested the impact of differentially antigen loaded and matured DC on NK cell activation of HCC patients. HCC patient $CD56^{lo}CD16^+$ NK cells showed increased level of activation (CD69 MFI and percent positivity) after coincubation with DC (3/4 patients, particularly with AdVhAFP/DC, Figure 4). However, the $CD56^{hi}CD16^-$ NK cells from HCC patients minimally (if at all) were activated by the different DC groups. Two of the patients (A3, B3) had $CD56^{hi}CD16^-$ NK cells which (according to MFI values) expressed high levels of CD69 without activation (also higher than the levels detected in other patients, Figure 1(a)), which were not further activated by DC. Overall, patient $CD56^{lo}CD16^+$ NK cells showed the ability to increase in CD69 expression, particularly after coincubation with AdV/DC as compared to NK alone, and other antigen-loaded DC produced more variable responses (Figure 4).

In order to determine any impact of DC antigen loading on Treg expansion *in vitro*, HD $CD4^+$T cells were cocultured for 6 days with different DC preparations. The pep/DC and prot/DC-stimulated groups showed increased $CD3^+CD4^+CD25^{hi}FOXP3^+$ cells, while both AdVhAFP/DC and pmAdVhAFP/DC-stimulated groups showed reduced Treg frequencies and FOXP3 expression levels, compared to baseline levels (Figure 5(a)). The opposite pattern was seen with the total $CD3^+CD4^+CD25^+$-activated T cell population, indicating that the AdV/DC were superior for overall activation of $CD4^+$ T cells (the frequency of $CD3^+/CD4^+/CD25^+$ cells), without expanding $FOXP3^+$ Treg. Using the same experimental method, three HCC patient cells were tested for Treg expansion. While patients A1 and B3 showed the same pattern as the HD, HCC patient A3 showed a different pattern, in which no DC group showed a relative reduction in Treg *in vitro* (Figures 5(b) and 5(c)). The total $CD3^+CD4^+CD25^+$-activated cells were tested in the patient cultures, and patients A1 and B3 again showed the same pattern as the HD, while patient A3 showed most activated cells in the prot/DC group (Figure 5(d)).

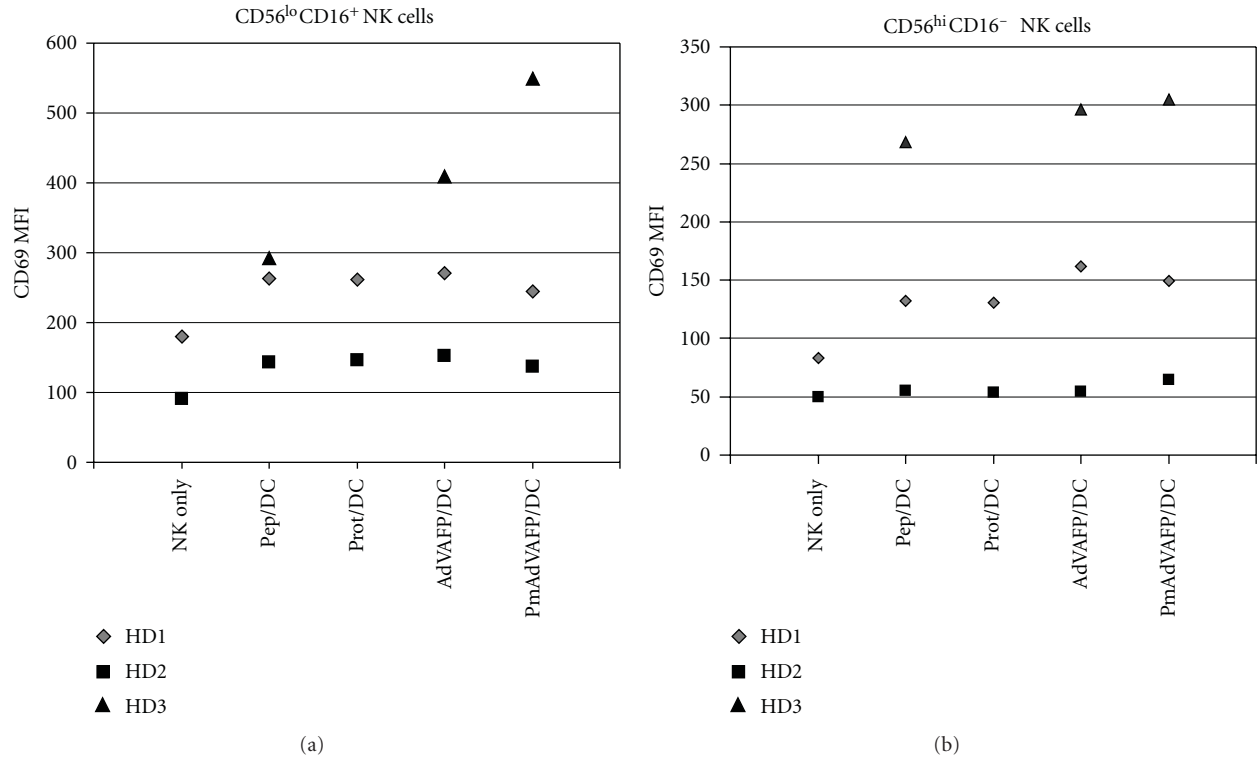

Figure 3: CD69 expression on healthy donor $CD56^{hi}$ and $CD56^{lo}CD16^{+}$ NK cells. Phenotyping of NK cells from HD PBMC after 48 hr coculture with different DC groups, showing CD69 upregulation on the (a) $CD56^{lo}CD16^{+}$ and (b) $CD56^{hi}CD16^{-}$ subsets for three HD.

3.5. Cytokine Production. In order to characterize the DC-lymphocyte (NK cell or $CD4^{+}$ T cell) interaction environment, cell-free supernatants were collected from the different HD and HCC patient cell coculture experiments. Supernatants were tested by multiplex Luminex assay to simultaneously assess the levels of cytokines, chemokines and growth factors, including: GM-CSF, IFN-γ, MCP-1, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IP-10 (Figure 6 and not shown). This allowed us to examine a functional readout of those interactions. IL-10 (produced by suppressive cells in some settings) was largely below the level of detection (5 pg/mL). Of the "Th1/effector" cytokines and chemokines in the cocultures of $CD4^{+}$ T cells and DC groups (IFN-γ, IP-10, TNF-α, IL-2), IFN-γ was notably higher in the two HD and one HCC (A1) coculture of $CD4^{+}$ with AdV/DC, as compared to the other DC conditions. IP-10 also showed an increase for $CD4^{+}$ T cells stimulated with AdV/DC (both HD and two patients: A1 and B3). Additionally, elevated IP-10 secretion was observed in prot/$DC^{+}CD4^{+}$ T cell cultures of HD1 and all three patients, but not to the level observed in AdV/DC cocultures. IL-2 expression, detected in all patients and only one HD, was only minimally influenced by DC and appeared $CD4^{+}$ T cell derived (data not shown). HD IFN-γ, IP-10, and TNF-α production was much higher than HCC patient levels (Figure 6(a)). Similar to TNF-α and IP-10, MCP-1 expression was greater in HD and was largely derived from immature DC and AdV/DC (reduced in pmAdV/DC, not shown). IL-5 expression was detected in one HD and two HCC cell coculture of $CD4^{+}$ with AdV/DC (not shown).

Coculture of NK cells with the differently antigen-loaded DC groups yielded minimal levels of IFN-γ and TNF-α and IP-10 which were largely restricted to HD cells and pmAdV/DC (Figure 6(b)). As above with the $CD4^{+}$ T cell cocultures, MCP-1 production was robust in all cocultures and DC derived (not shown). IL-6 was restricted to HD and pmAdV/DC cocultures (not shown). Lastly, IL-8 were broadly detected in most groups and were more highly expressed in HD cultures than HCC cultures (not shown). These data highlight important functional differences between HD and HCC cells, with reduced cytokine and chemokine production levels (but some similar trends) among the HCC patients.

4. Discussion

Immunotherapy holds potential for treatment of hepatocellular carcinoma, as few effective treatments are available, and immunotherapy vaccine strategies have largely shown immunogenicity and less toxicity than current chemotherapy [40–44]. We have previously conducted vaccination clinical trials of AFP-based vaccines for HCC, with a goal of activating the immune system against cells expressing the AFP oncofetal antigen. Our current investigation was undertaken with two aims. First, we sought to define phenotypic changes in NK cells and Treg over time in the patients treated with an

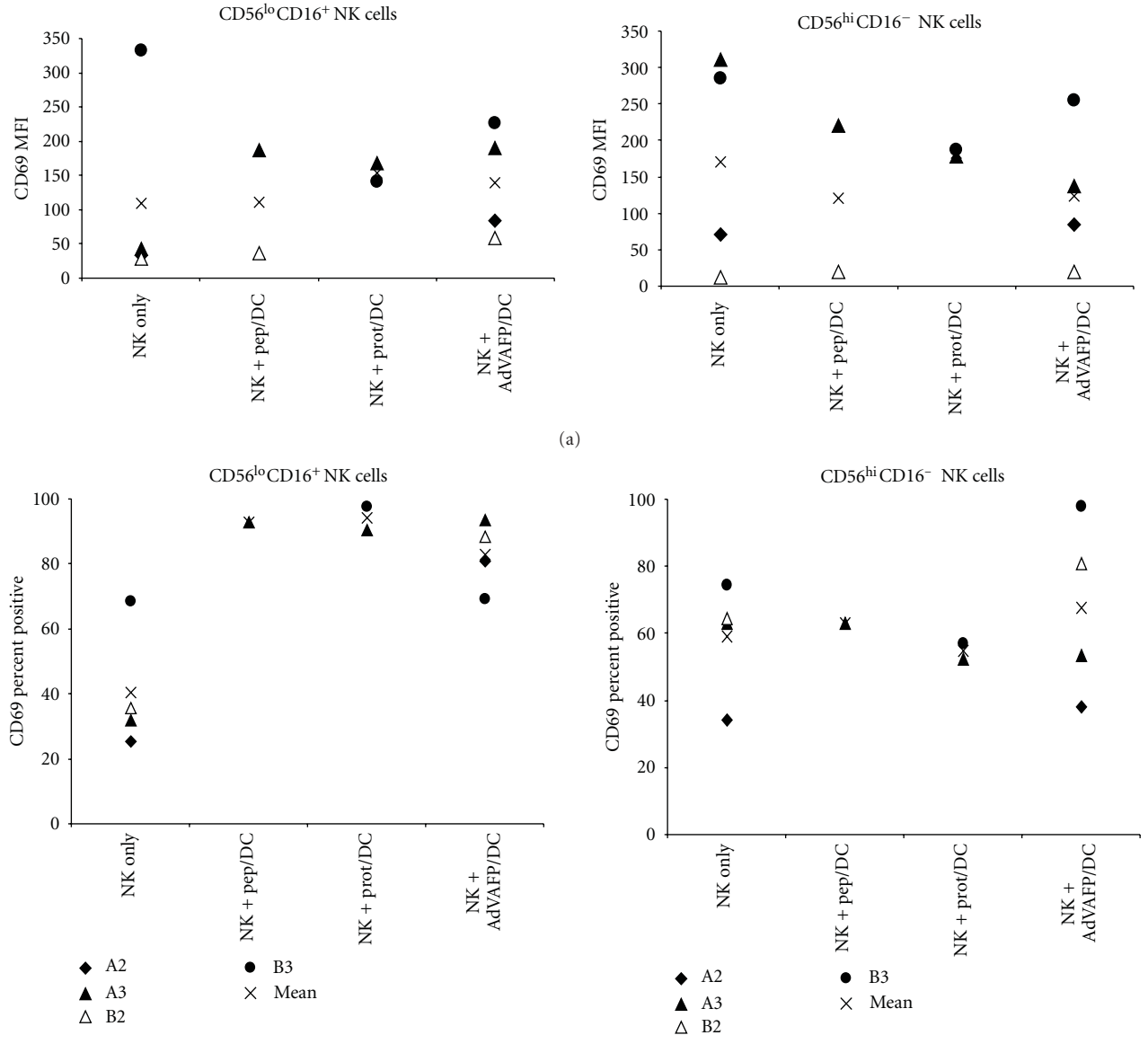

Figure 4: CD69 expression on HCC patient NK cells. Phenotyping of NK cells from HCC patients after 48 hr coculture with different groups of DC, showing CD69 upregulation on $CD56^{hi}CD16^{-}$ and $CD56^{lo}CD16^{+}$ subtypes, by MFI (a) and percent positivity (b).

AFP pep/DC vaccine, for the first time. Second, we tested *in vitro* responses of NK cells and Treg to DC vaccines including peptide pulsed, protein-loaded, and AdV-transduced, to determine the DC antigen loading and maturation strategy which would promote NK activation and minimize Treg expansion. Such data are critical for the design of next generation DC vaccines with broad immunologic impact on both effectors and suppressive mechanisms.

An effective vaccine against HCC would activate not only tumor antigen-specific adaptive immune responses, but also innate NK cell effectors to crosstalk with DC, promote type I responses, and potentially also directly kill HCC cells. In addition, downregulating Treg cells would help to minimize immunosuppression and potentially allow enhanced antitumor effector function. By testing PBMC from the peripheral blood of patients treated with the AFP pep/DC vaccine, we found evidence for activation of NK cells in most patients, as shown by increase in CD69 and CD25 expression. We also found evidence for downregulation of Treg cells in most patients, as shown by decreased FOXP3 expression in those $CD4^{+}CD25^{+}$ T cells. These results illustrate the possibility of rationally modulating the immune system with DC to increase anti-HCC immunity. While additional functional assays of NK cell killing and Treg suppression would have strengthened our report, there were insufficient banked PBMC remaining for such assays.

In this data set, A1, A4, and B2 received 10^6 DC/vaccine, and B5 and B8 received 5×10^6 DC/vaccine, and all were

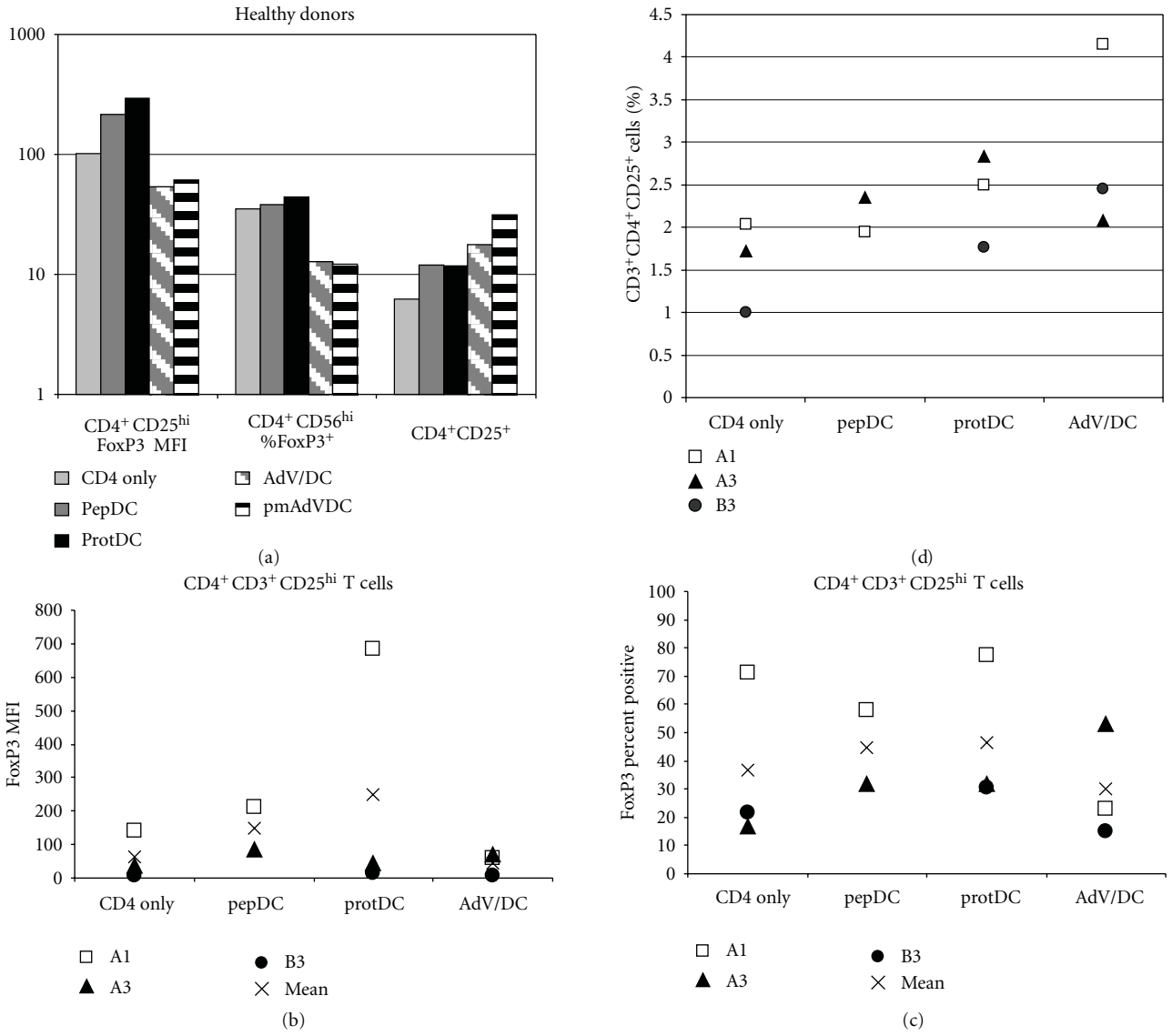

Figure 5: Treg cell responses to DC coculture. HD (a) and HCC patient (b, c, and d) $CD4^+$ T cells were cocultured with differentially treated DC. The FOXP3 expression in $CD3^+/CD4^+/CD25^{hi}$ cells is shown in (a) as MFI (left), percent positive (middle). The right group is the frequency of total activated ($CD25^+$) $CD4^+$ T cells. The FOXP3 MFI in Treg in HCC patients is shown in (b). The percent $CD3^+/CD4^+/CD25^{hi}/FOXP3^+$ Treg in HCC patients is shown in (c). The overall frequency of activated $CD4^+$ T cells is shown in (d) (% $CD3^+/CD4^+/CD25^+$ cells).

stage IV (Table 1). Because A1 showed both NK cell activation and Treg FOXP3 decrease and B2 showed neither, there does not appear to be an overt DC dose effect for these assays. A1, B5, and B8 were heavily pretreated with a variety of chemotherapies, while B2 had only surgery before the DC vaccines, hence the chemotherapies do not appear to absolutely preclude NK cell and Treg changes observed [26].

By testing *in vitro* responses to a variety of DC vaccines, we were able to assess their comparative ability to stimulate NK and Treg cells. We found that of the antigen-loading strategies and maturation treatments we tested, AdV/DC tended to activate NK cells more than pep/DC or prot/DC as measured by CD69 expression. In addition, HD cells showed downregulation of FOXP3 in $CD4^+CD25^{hi}$ Treg cells in the presence of AdV/DC (or pmAdV/DC) as compared with pep/DC or prot/DC. These AdV/DC groups also promoted increased $CD3^+CD4^+CD25^+$ total activated T cells. When testing DC groups with HCC patient cells, the levels of NK cell activation, and differences between groups, were weaker. While our sample size was small, these data highlight the different outcomes from advanced stage HCC patients. This may also indicate an NK cell function defect in these advanced stage patients [26], as noted by others [22]. The Treg assessments also indicate that the general trends observed in HD samples could be similar to HCC patient cells, but there were weaker responses and exceptions. Overall, these results support our conclusions from the phenotyping of vaccinated HCC patient NK and Treg cells, that the immune system can be favorably modulated in multiple ways by the DC vaccines. Furthermore, they

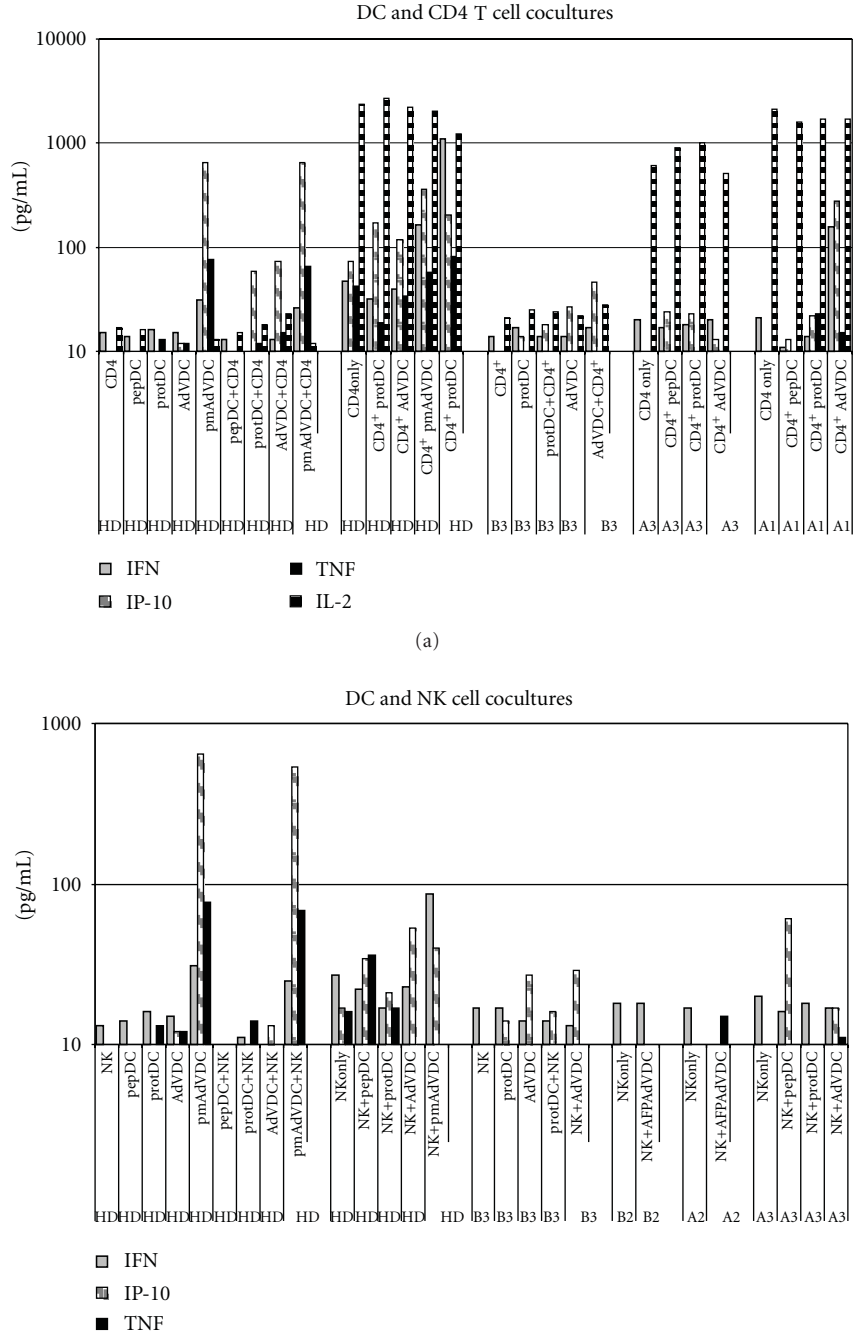

Figure 6: Luminex results: production of chemokines and cytokines. Graphs are grouped according to scale of cytokine production and function. (a) IFN-γ, IP-10, TNF-α, and IL-2 production after $CD4^+$ DC coculture. (b) MCP-1 and IL-5 production after $CD4^+$ DC coculture.

indicate that an AdV/DC vaccine may be superior to peptide or protein loaded DC, although patient-specific differences should be anticipated. In future studies, we will also test prematured AdV/DC with patient-derived cells, as our banked samples were in insufficient numbers to test all DC groups in all patients.

Cytokine production in response to DC vaccine coculture is a functional measure of activation of NK and Treg cells. It was interesting to note that cytokines and chemokines tested for by Luminex were produced more abundantly by HD-derived cells than HCC-derived cells. This again highlights the difficulty of inducing an antitumor immune response in HCC patients, and suggests that additional immune stimulatory and immune suppression reducing efforts may be required to promote the desired antitumor immunity *in vivo*. The AFP antigen may also play a role in these responses. We have observed similar DC phenotypic maturation effects after AdV transduction

regardless of transgene; however, some specific molecular changes have been observed. We recently demonstrated that AdV/DC activate NK cells via transmembrane TNF and transpresented IL-15 [17]. In order to promote that DC-NK cell contact, we have also found that AdV/DC produce IL-8 and IP-10, which cause chemotaxis of NK cells towards DC (Vujanovic and Butterfield, submitted 2011). In this study, we find that the HD cocultures produced more IL-8 and IP-10 (not shown and Figure 6) than the HCC cocultures.

We have performed additional preliminary studies comparing AdVLacZ and AdVhAFP in HD DC, and we find a reduction in DC surface transmembrane TNF expression (but similar *trans*presented IL-15) with AdVhAFP. These data support the conclusion that preclinical studies to develop more effective AFP-based immunotherapy approaches for HCC should utilize patient cells, and that additional compensatory manipulations may be required to fully activate NK cells in addition to antigen-specific T cells, while limiting Treg expansion.

5. Conclusions

In conclusion, we find that DC-based vaccines can modulate not only antigen-specific T-cell responses, but also innate effectors and counter-regulatory mechanisms. Optimal antigen loading of DC and maturation signaling may allow for development of DC vaccines which will subsequently deliver specific signals to the broad array of tumor-reactive cells they encounter *in vivo*. Immunotherapy of cancer has the potential to improve treatment for many cancers, and this investigation into NK cell activation and Treg modulation induced by DC vaccines against HCC is a step forward for designing the next generation of DC vaccines.

Abbreviations

HCC: hepatocellular carcinoma
DC: dendritic cells
AFP: Alpha-fetoprotein
AdV: adenovirus
MFI: mean fluorescence intensity
Treg: regulatory T cells
PBMC: peripheral blood mononuclear cells.

Disclosure

L. H. Butterfield is coinventor of patents covering aspects of AFP as a target for T cell-mediated anti-HCC immunity.

Acknowledgments

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Research Article

Evaluation of Epstein-Barr Virus Latent Membrane Protein 2 Specific T-Cell Receptors Driven by T-Cell Specific Promoters Using Lentiviral Vector

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Transduction of latent membrane protein 2 (LMP2)-specific T-cell receptors into activated T lymphocytes may provide a universal, MHC-restricted mean to treat EBV-associated tumors in adoptive immunotherapy. We compared TCR-specific promoters of distinct origin in lentiviral vectors, that is, Vβ6.7, delta, luria, and Vβ5.1 to evaluate TCR gene expression in human primary peripheral blood monocytes and T cell line HSB2. Vectors containing Vβ 6.7 promoter were found to be optimal for expression in PBMCs, and they maintained expression of the transduced TCRs for up to 7 weeks. These cells had the potential to recognize subdominant EBV latency antigens as measured by cytotoxicity and IFN-γ secretion. The nude mice also exhibited significant resistance to the HLA-A2 and LMP2-positive CNE tumor cell challenge after being infused with lentiviral transduced CTLs. In conclusion, LMP2-specific CTLs by lentiviral transduction have the potential use for treatment of EBV-related tumors.

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus associated with many human malignancies including a subset of Hodgkin disease, Burkitt lymphoma, nasopharyngeal carcinoma (NPC), and some gastric carcinomas [1–5]. The malignancies associated with EBV can be grouped into 3 types according to the latency gene expressional profiles [4–6]. In NPC, the EBV proteins expressed are EBNA1, latent membrane protein 1 (LMP1), and LMP2 [7]. It has been shown that all 3 antigens can induce $CD8^+$ cytotoxic T lymphocytes (CTLs), which play roles in antitumor immune response [8, 9]. However, only weak responses against EBNA1 can be detected in some individuals and the phenotypic and functional analyses of these induced EBNA-1-specific T cells revealed that EBNA1 is presented to $CD4^+$ T helper as well as Treg cells, which suppress the antiviral immune response. Moreover, the poor immunogenicity of EBNA1 has also been attributed to the presence of a Gly-Ala repeat (GAr) sequence, which prevents the presentation of EBNA1-derived antigenic peptides by MHC class I molecules. This GAr-mediated function has been linked to its capacity to prevent EBNA1 synthesis, as well as proteasomal degradation [10–13]. On the other hand, LMP-1 is the only EBV protein with recognized oncogenic activity that can transform normal cells into malignant ones, thereby limiting its application as a potential immunotherapeutic target. Additionally, the LMP1-specific CTL frequency is low, and the reactivation of LMP1-specific CTL lines has been shown very difficult, in part because LMP1 is toxic when expressed at high levels. In NPC, LMP2 offers the best opportunity for specific targeting since it is consistently expressed and

the T-cell determinants in LMP2 sequence have been well defined [14–17]. Many studies, including clinical trials, have proven LMP2 to be an ideal immunotherapeutic target and inducer, which so far has not shown oncogenicity [16, 18–20]. It has been shown that this antigen can be processed by a proteasome system. The peptides are engaged in the major histocompatibility complex (MHC) class I, then move to the cell surface, and migrate to the $CD8^+$ T cells on the cell surface [21–24]. Many $CD8^+$ T cell-recognizing epitopes have been identified and most of them are conserved in NPC cells among different populations. Low LMP2 is a widely used targeting molecule and antigen for the immunotherapy of type II EBV-associated malignancies [19, 21, 25–29].

Adoptive immunotherapy with CTLs holds great promise for the treatment of cancer. Among them, the treatment of EBV-associated tumors has by far shown the most success [26–28, 30, 31]. With the development of molecular and cellular biology, tumor-specific CTLs can be selected and the T cell receptor genes can be cloned into highly efficient viral vectors for transfer into the patient's T cells. This concept has been utilized since 1999, when Clay et al. transferred lytic function by retroviral vectors encoding the α and β chains of the TCR against EBV-associated tumors [32]. Today many practitioners have designed and applied these engineered CTLs for the treatment of various human malignancies [33–37]. However, the efficacy and efficiency of this application still needs to be optimized, especially when using retro- or lentiviral vectors for TCR transduction. These vector systems can integrate transgenes into chromosomes that have the potential to "immortalize" a normal cell. Thus, a specific T-cell promoter that can be activated only in T cells becomes critical for safety concerns. At the same time, transducing efficiency should be considered when using both these viral vectors.

In our investigation, we used an HLA-A2-restricted EBV LMP2-specific TCR, TCR5.05, to compare 4 different kinds of T-cell-specific promoters: Luria, Delta [38], Vβ5.1 [39], and Vβ 6.7 [40]. Our results show that all these T-cell-specific promoters can drive the transcription of the TCR gene without changing the transduced T cell phenotypes. We also found that CTLs generated by a lentiviral vector containing specific promoters and TCR genes can lyse target cells specifically. We further evaluated the CTLs in vivo and found that they can retard the growth of EBV-LMP2 expressing tumors and prolong the life of tumor-bearing mice. We reported for the first time that Vβ 6.7 promoter is most efficient when using the lentiviral vector to transduce T cells for targeting HLA-A2-restricted EBV-LMP2 antigens. This study may be helpful in designing and developing novel TCR-based adoptive immunotherapy for the treatment of EBV-associated tumors.

2. Material and Methods

2.1. Animals and Cell Lines. Six- to 8-week-old nude mice were purchased and maintained in the SPF animal facility at Nanjing Medical University. All procedures used in this study complied with institutional policies of the Animal Care and Use Committee of Nanjing Medical University.

The cell lines used in these experiments included 293T, HSB2 (human leukemic T-cell line); HLA-A2 restricted, Epstein Barr virus-transformed B lymphoblastoid cell lines (LCLs); K562; CNE (nasopharyngeal carcinoma cell line); T2 cell lines (deficient in TAP but still express low amounts of MHC class I on the surface of the cells, kept in the author's lab). In animal model, CNE cells stably expressing HLA-A2 and LMP2 were established by plasmid pIRES/HLA-A2/LMP2 transfection and selection. All cell lines were cultured in RPMI 1640 plus 10% fetal bovine serum, L-glutamine, nonessential amino acids, and penicillin-streptomycin (100 U/mL) (Invitrogen).

2.2. Construction of Lentiviral Vector Plasmids. TCR plasmid PL5.05 and 4 T lymphocyte-specific promoters (PSK-Delta/Vβ 5.1/Luria/Vβ 6.7) were kindly provided by Rimas Orentas from Medical College of Wisconsin. EBV LMP2-specific TCR cDNA we used was obtained from T-cell clone which was generated by incubating HLA-A2-restricted PBMC with peptide (CLGGLLTMV, LMP2: 426–434) as described by Orentas et al. [36]. TCR PL5.05 α and β chains were amplified by PCR using PL5.05 as a template and cloned into 4 lentiviral vectors containing various 4 T-cell promoters constructed from the parent PWPT-GFP vector. The primers were α chain: Primer1A: CAACGCGTCGGAATTCAGGCTCTCTTG; Primer2A-3A: GTCATCGTCTTTGTAGTCGCTGGACCACAGCCGC; CAGGTCGACTCACTTGTCGTCATCGTCTTTGTAGT; β chain: Primer1B: ACTACGCGTCACCATGGCTATAGT-GTCTCTAGATCAAAG; Primer2B-3B: TTCTGAGATGAG-TTTTTGTTCCTAAAGGGAACAAAAGCTGGAAGTCGA-CTCAATTCAGATCCTCTTCTGAGATGAGTTT. The α and β were linked with Flag and Myc tag, respectively. All the amplicons were sequenced and cloned into Mlu I and Sal I sites of PWPT-GFP vectors.

2.3. Lentivirus Production. Lentiviruses were prepared by transient transfection of 293T cells, using a liposomal cotransfection method. To summarize, the 293 T cells were seeded at 1×10^7 cells per 10-cm plate. The cells were transfected 12–16 hours later with 20 μg lentiviral transfer vector, 12 μg Delta 8.9, and 18 μg VSV-G envelope plasmids 8.91 (Delta 8.9 and VSV-G envelope plasmids are helper lentiviral plasmid which are used for packing lentivirus). Forty-eight to 72 hours later, the supernatant was collected, centrifuged to remove the cellular debris, and concentrated approximately 30-fold by ultracentrifugation.

2.4. Determination of Lentiviral Titer. Titers of concentrated lentivirus encoding green fluorescent protein (GFP) were determined by serially diluting and infecting 293T cells by the polybrene transduction method as previously described [41]. Titers (transducing units (TUs) GFP-positive cell dilution factor) of the lentiviral vectors ranged from 10^6 to 10^7 TU/mL.

2.5. Transduction of PBMCs and T Cells. Peripheral blood monocytes (PBMCs) were from an HLA-A2, healthy human.

T cells were obtained from anti-CD3 conjugated magnetic beads (Miltenyi Biotec, Bergish Glad-bach, Germany). The PBMCs and T cells were cultured in AIM-V and interleukin-2 (IL-2; PeproTech, Rocky Hill, NJ, USA) at 300 IU/mL. For OKT3 stimulation, the cells were placed initially in either a medium with anti-CD3 antibody, OKT3 (Ortho Biotech, Bridgewater, NJ, USA) at 50 ng/mL or in an OKT3 medium after transduction at the initial changing of the culture medium in the presence of IL-2. For transduction of the PBMCs or T cells, 1×10^6 cells were adjusted to a final volume of 1 mL in a 24-well, tissue culture-treated plate with the viral supernatant and Polybrene (8 mg/mL; Sigma, St. Louis, Mo, USA). The cells were transduced by centrifugation of the plates at 1000 g for 1.5 hours at 32°C. The plates were placed in a 37°C, humidified, 5% CO_2 incubator overnight, and the medium was replaced the next day.

2.6. Flow Cytometric Analysis. CD3 expression on cell surface was measured with allophycocyanin-conjugated antibodies and the corresponding isotype controls (BD Biosciences). TCR PL5.05 staining was performed by using anti-TCR α chain antibody (prepared from our lab) followed by phycoerythrin (PE)-labeled second antibody. Cells were stained in a FACS buffer made of PBS (Invitrogen, Carlsbad, Calif, USA) and 0.5% bovine serum albumin. Cells were collected with a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, Calif, USA) and analyzed using CellQuest software (BD Biosciences).

2.7. Real-Time PCR. After 3 days, total RNA was extracted from the HSB2 cells which have been infected with lentivirus containing EBV-LMP2-specific TCR α and β chain driven by Luria, Delta, Vβ 5.1, and Vβ 6.7 T-cell-specific promoters. cDNA was reverse transcripted by a high-capacity cDNA reverse transcription kit (ABI, Foster, Calif, USA) using random primers. For α chain SYBR forward primer: 5′-ctttcaaaacctgtcagtgattgg, reverse primer: 5′-cagcgtcatgagcagattaaacc. For β chain SYBR forward primer: 5′-ggccaccttctggcagaac, reverse primer: 5′-agagcccgt agaactg-gacttg. Real-time PCR with SYBR dyes was performed on an ABI 7900 real-time machine and analyzed by SDS2.4 software.

2.8. Western Blotting. Fifty micrograms of total protein from each sample was loaded for SDS-PAGE and subsequently transferred onto the PVDF membranes. After blocking, the membranes were hybridized with anti-Flag and Myc tag antibodies, respectively. The membranes were washed and incubated with secondary antibody, followed by developing.

2.9. Measurement of Lymphocyte Antigen Reactivity. Target cells were prepared by using T2 cells pulsed with peptides (10 ng/mL) in cell culture medium or tumor cell lines for 2 hours at 37°C and then washed twice in PBS. $CD8^+$ T cells were isolated from lentiviral transduced PBMCs using anti-CD8 beads from Miltenyi Biotec according to the manufacturer's protocol. For the assay, effector cells ($CD8^+$ T cells) and target (peptide-pulsed T2 or tumor cells) were incubated in a 0.2-mL culture volume in the wells of a 96-well culture plate at E : T = 50 : 1, 25 : 1, and 5 : 1. The cells were cocultured for 18 hours, and the supernatant was harvested. The supernatants were analyzed for interferon (IFN)-γ secretion, using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Bender Medsystem, Vienna, Austria). The supernatants were also measured for LDH levels using a commercially available kit (Roche, Boehringer Mannheim, Germany).

2.10. Mouse Immunization and Tumor Challenge. Tumor-bearing model were established by injecting 1×10^6 HLA-A2 and LMP2-positive CNE cells subcutaneously in the flank of six- to 8-week-old nude mice. The mice were infused with transduced CTL via tail vein 1 week after tumor cell injection weekly for a total of two weeks. Mice immunized with the mock or saline were used as a control. Tumor diameter was measured by calipers twice per week and recorded as the mean of narrowest and longest surface length for each animal in the group. Mice were sacrificed when the tumor size reached a 20 mm average diameter. Each experiment was performed at least twice, and results were essentially similar unless described otherwise.

2.11. Statistical Analysis. Data are expressed as mean ± standard error of mean (SEM), as indicated in each experiment. And the comparisons between the groups were made by one-way ANOVA followed by unpaired t-test. A 4.0 version (2005) of the GraphPad Prism software was used for this purpose. Values of $P < 0.05$ were considered significant. Survival curves were estimated using the Kaplan-Meier method, and significance was assessed using the log-rank or the χ^2 test.

3. Results

3.1. Vβ 6.7 Promoter Is the Most Optimal for TCR Expression. The map of lentiviral vector pWPT-promotor-α/β chain and the schematic diagrams representing the structures of the lentiviral vectors are shown in Figure 1. The promoter-α/β chain was amplified by PCR and inserted between *Mlu*I and *Sal*I sites. The mock vector contains TCR α/β chain without any T-cell-specific promoter. TCR expression under four T-cell-specific promoters was compared by using real-time PCR, as shown in Figure 2(a). The HSB2 cells were incubated for 24 hours in medium and then exposed to each vector at a multiplicity of infection (MOI) of 10. Three days aftertransduction, the T cells were analyzed by real-time PCR and Western blotting. We observed that all the lentiviral vectors were able to transduce the T cells, using the Luria, Delta, Vβ 5.1, and Vβ 6.7 promoter-containing vectors. The Vβ 6.7 promoter vector had the highest TCR at transcriptional level. When the normalized α and β chain mRNA levels of the Luria promoter group were set at 100.03 ± 21.09 and 68.45 ± 23.75, Delta was 46.15 ± 11.01 and 26.54±6.86, Vβ 5.1 was 42.08±6.78 and 28.76±19.75, and Vβ 6.7 was 150.58 ± 32.02 and 102.564 ± 17.75, and mock was

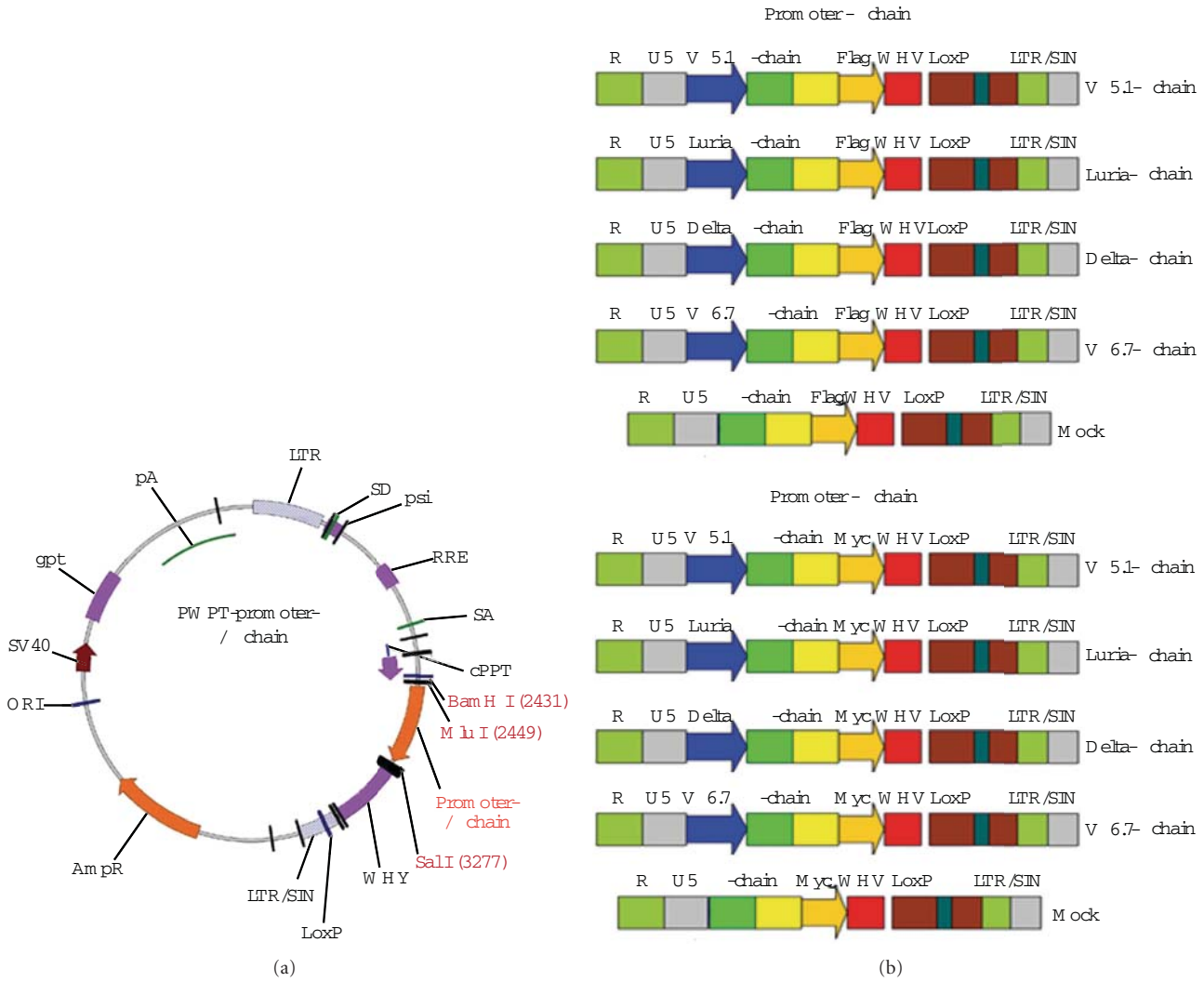

Figure 1: The map of lentiviral vector pWPT-promotor-α/β chain and the schematic diagrams representing the structures of the lentiviral vectors. (a) The map of lentiviral vector pWPT-promotor-α/β chain. The promoter-α/β chain was amplified by PCR and inserted between *Mlu* I and *Sal* I sites. (b) The schematic diagrams representing the structures of the lentiviral vectors. All the α/β chains of the anti-LMP2 TCR PL5.05 were driven by individual T-cell-specific promoter except mock which contains only α or β chains without any promoter region. Promoter-α chains in diagram forms were the lentiviral vectors designed to express α chain driven by Vβ 5.1, Luria, Delta, and Vβ 6.7 promoters, respectively. Promoter-β chain used Vβ 5.1, Luria, Delta, and Vβ 6.7 promoters to produce the individual β chain.

4.89 ± 3.09 and 4.08 ± 2.98. The mRNA levels of TCR were consistent with the protein levels used in Western blotting to detect the protein levels of the TCR α and β chain. Protein levels of TCR were much higher in the Vβ 6.7 group than in the other groups (Figure 2(b)). The expression of TCR α and β chain on the HEK293T cells and HepG2 cells (human hepatocellular carcinoma cell line) were almost not detected (Data not shown). These results suggest that the lentiviral vectors can express TCR in the T cell lines and PBMCs. Four different promoters have different levels of capacity to drive TCR expression.

3.2. Lentiviral Vectors with Various Promoters Can Transduce T Cells Efficiently. HSB2 and PBMCs were infected with lentiviral vectors having various promoters expressing the TCR α and β chain at MOI = 1 or 10. Three days after infection, expression of the TCR α chain was detected in the $CD3^+$ T cells by FACS with a Flag tag antibody. At MOI = 1, the TCR α chain positivities from $CD3^+$ cells were 16.76 ± 4.62%, 34.15 ± 3.71%, 42.08 ± 6.03%, and 58.58 ± 5.02% under Luria, Delta, Vβ 5.1, and Vβ 6.7, respectively. At MOI = 10, the positive TCR α chain was 23.42 ± 10.63%, 47.14 ± 4.53%, 46.33 ± 2.96%, and 60.46 ± 5.41%, under Luria, Delta, Vβ 5.1, and Vβ 6.7 T-cell-specific promoters of $CD3^+$ cells, respectively (Figure 3(a)). The Vβ 6.7 group had the highest transducing efficiency, as evidenced by means of 58.58% and 60.46% positive at MOI = 1 or 10. We next checked the LMP2-TCR expression by flow cytometric analysis. As shown in Figure 3(b), 51.3% or 62.1% of the HSB2 or PBMC cells, respectively, were

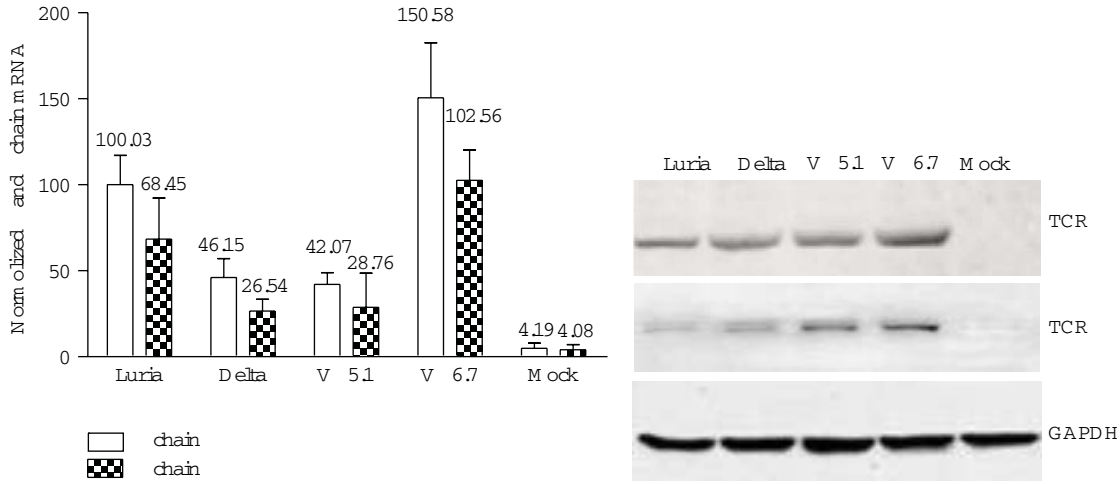

Figure 2: Comparison of lentiviral vector constructions driven by different T-cell-specific promoters. (a) Comparison of mRNA level of TCR PL5.05 α and β chain under different T-cell-specific promoters by using quantitative RT-PCR. The number on each column corresponds to the mean number of mRNA normalized by GADPH mRNA, and the vertical bar represents the SD. (b) Conventional western blot assays from HSB2 cells which are infected with lentivirus containing TCR α and β chains driven by Vβ 6.7, Delta, Vβ 5.1, and Luria promoters. Either Flag or Myc fusion proteins were transferred to membranes and incubated with the indicated antibody. Approximately, fifty micrograms of each protein per lane were applied for electrophoresis. Equal protein loading was controlled by staining of GAPDH (lower panel). Statistical analysis was determined using the Student t-test with $^*P < 0.05$, compared to other groups.

positive when confirmed by the result of FACS. The empty lentiviral vector-infected control group showed no TCR 5.05 expression. To test the stability of TCR expression on the surface of the cytoplasm membranes, we checked the expression of TCR 1 and 7 weeks after transduction by using FACS. Our experiments showed that there were no significant changes in the TCR expression levels in either the HSB2 cells or the $CD3^+$ T cells transduced by all 4 lentiviral vectors. However, the Luria group had the lowest, and the Vβ 6.7 group had the highest transduction efficiency 1 week and 7 weeks after transduction (Figures 3(c) and 3(d)). The percentage of TCR-positive cells in $CD3^+$ group 1 week after transduction is similar to that of the 3-day transduction experiment described above.

3.3. Transduced PBMCs Can Specifically Lyse HLA-A2/LMP2, Expressing Target Cells. To assess the recognition of tumor antigens by lentivirus-transduced PBMCs and $CD8^+$ T cells, the cells were cocultured with the indicated tumor cell lines or T2 cells pulsed with $LMP2_{426–434}$ (CLGGLLTMV) (CLGG). After sorting, the $CD8^+$ cells were collected and incubated with target cells at effector-to-target-cell ratios (E : T) = 50 : 1, 25 : 1, and 5 : 1. As shown in Figure 4(a), the Vβ 6.7 group has the highest lytic activity when using all 3 E : T ratios. To test the specificity of cytotoxicity, we chose the Vβ 6.7 lentiviral vector infected with PBMCs and $CD8^+$ groups against different targeting cells. The results showed that Vβ 6.7 lentiviral vector-infected PBMCs could lyse T2-CLGG and LCLs effectively moderately but could not lyse T2 cells, T2 cells loaded with nonrelated peptides (T2-LLWT), and K562 cells (Figure 4(b)).

We also measured the IFN-γ levels in the supernants of the transduced-PBMC cytotoxicity experiments. All 4 promoter-containing lentiviral vector groups which transduced PBMCs secreted high levels of IFN-γ (>500 pg/mL) when incubated with CLGG and LCLs but secreted very low levels of IFN-γ when incubated with T2, T2 LLWT, or K562 cells (Figure 4(c)). These results further confirmed that the lysis is specific.

We next tested the cytotoxicity of Vβ 6.7-transduced $CD8^+$ T cells against the targeting cells described above. Similar to the result involving PBMCs, the transduced $CD8^+$ T cells had a higher cytotoxicity against the T2-CLGG and LCL, but minimal effects on T2-LLWT, T2, and K562 cells (Figure 4(d)). LCLs are EBV-transduced B lymphocytes which belongs to type III infection, expressing nine EBV genes encoded by the virus including LMP2. The results indicated that Vβ 6.7 lentiviral transduced T cells can specifically lyse HLA-A2-restricted tumor cells expressing EBV-LMP2.

4. Transduced $CD8^+$ Cells Can Slow the Growth Rate of LMP2-Expressing CNE Tumors in Mice

CNE tumor cells stably expressing HLA-A2 and LMP2 were inoculated subcutaneously at 5×10^5 cells per mouse to establish the tumor model. Ten days later, the peptide-pulsed, lentiviral vector-transduced $CD8^+$ cells were infused via the tail vein. The tumors were monitored daily till the tumor reached 1 cm^2, when the mouse was sacrificed. Each group of the transduced $CD8^+$ cells was shown to slow or abolish

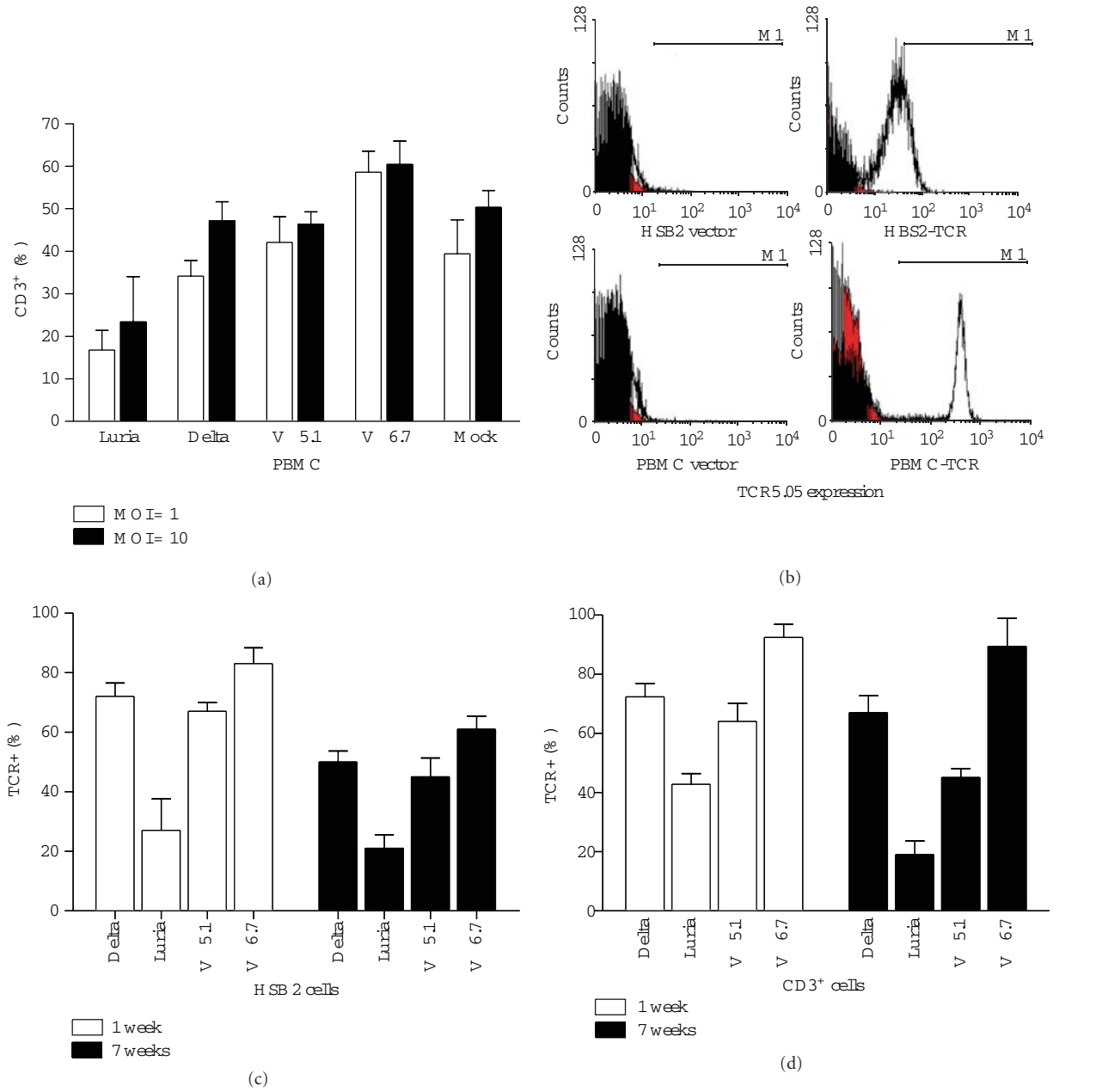

Figure 3: Expression of TCR α and β chains in the HSB2 cells and PBMCs. (a) The PBMCs were incubated with lentivirus containing EBV-LMP2 TCR α and β chains driven by T cell-specific promoters at MOIs of 1 and 10. Three days aftertransduction, cells were collected and stained for CD3 mobilization as a measure of TCR α chain expression. (b) Photographs of flow cytometric analysis for TCR α chain expression in HSB2 cells (top) and PBMC cells (bottom). The cells were infected with recombinant lentivirus or control empty lentivirus at MOI of 10 for 3 days. TCR staining was performed by using anti-TCR α chain antibody (prepared from our lab) followed by PE-labeled second antibody. (c) Percentage of TCR α chain-positive cells in transduced HSB2 cells 1 week and 7 weeks after infection at MOI of 10. (d) Percentage of TCR α chain-positive cells in transduced $CD3^+$ cells 1 week and 7 weeks after infection. PBMCs stimulated with IL-2 plus OKT3 for 24 hr were infected with the lentivirus at an MOI of 10. After 1 week, the PBMCs were analyzed by FACS for TCR α chain expression and then maintained in culture with IL-2 for 7 weeks for reanalysis for TCR α chain-positive cells.

the established tumors in the mouse model (Figure 5(a)). There were no statistically significant differences between the antitumor effects of the 4 promoter groups. All immunized groups were significantly different when compared with the saline and mock groups (Figure 5(b)). The mice were deemed dead when the tumor reached 1 cm^2. None of the mice in the Vβ 6.7 group died, and only 1 mouse died in each of the Luria, Delta, and Vβ 5.1 groups. All the mice in the saline group died 36 days after inoculation. These results demonstrated the therapeutic effects of reinfused CTL transduced with lentiviral vectors containing the specific TCR.

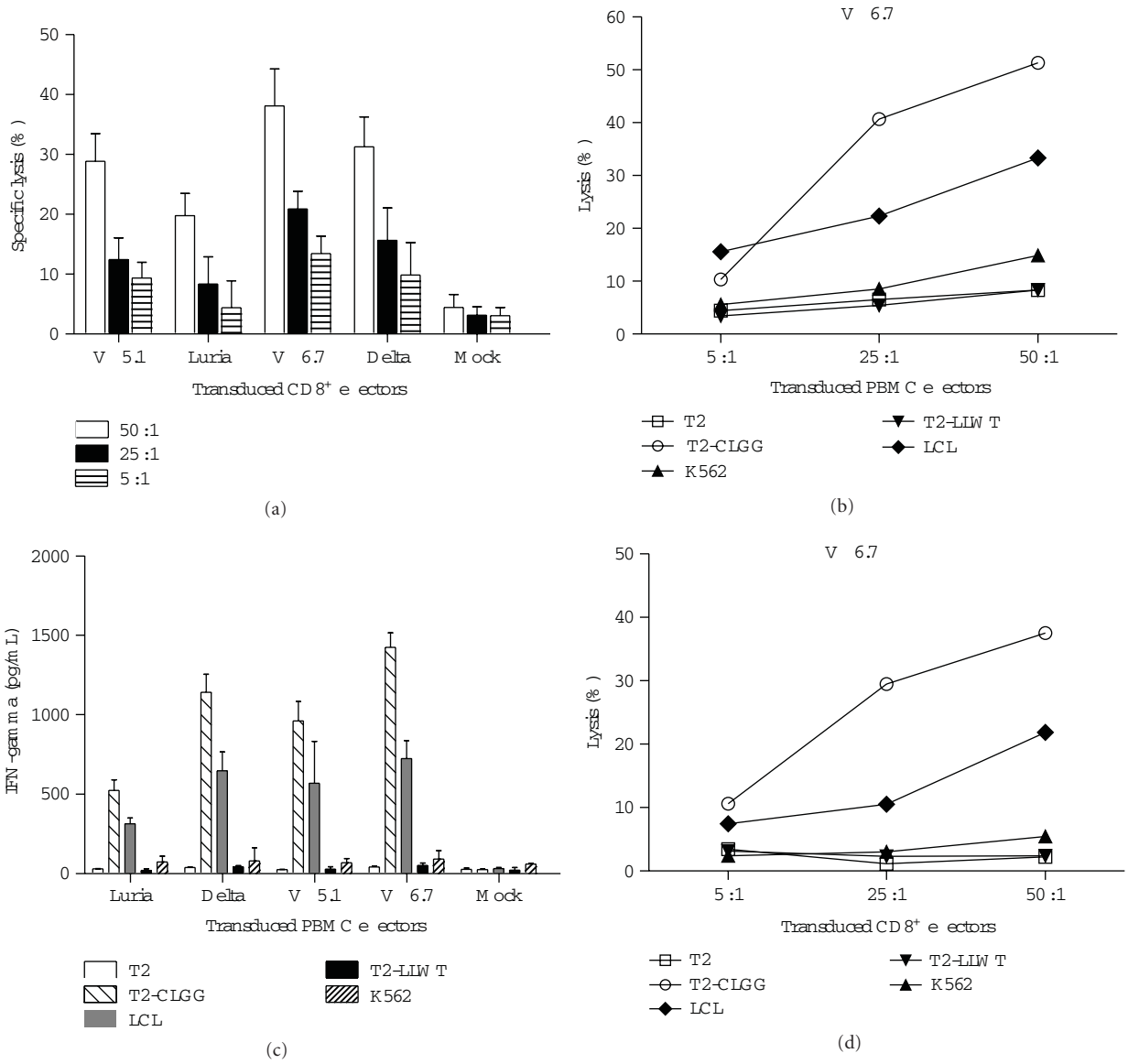

Figure 4: Transduced PBMCs specifically lyse HLA-A2-restricted LMP2-expressing target cells. (a) Lytic activity of $CD8^+$ cells selected from PBMCs transduced with lentivirus containing LMP2-TCR at indicated effector-to-target cell ratios (E : T) was demonstrated in an LDH-release assay. Targets were HLA-A2-restricted T2 cells loaded with CLGG. Results are expressed as percent of the value measured in control cells incubated with the same volume of medium (mean ± SD of 3 replicates). Lytic activity of PBMCs (b) or $CD8^+$ cells (d) selected from PBMCs transduced with lentivirus-containing LMP2-TCR driven by T-cell promoter Vβ 6.7 at indicated E : T was demonstrated in an LDH-release assay. Targets were HLA-A2-matched T2, T2-CLGG, LCL, T2-LLWT, and exceptional K562 cells. All figures are representative of 3 or more experiments using the same PBMC donor. (c) Levels of IFN-γ being released into the media from transduced PBMC effectors in the lysis assay above. PBMCs expressing LMP2-TCR were cocultured for 16 hr with the indicated target cells. The concentration of IFN-γ secreted into the medium was measured in an ELISA kit.

5. Discussion

Adoptive T-cell immunotherapy remains an active area in the correction of birth defects and the treatment of malignancies [26–28, 30, 31]. Unlike traditional immunotherapeutic approaches such as use of vaccine or antitoxin, adoptive T-cell immunotherapy is specific, repeatable, and much more effective. Adoptive T-cell therapy has advanced from simple ex vivo expansion of therapeutic T cells to gene-modified T cells. As the most important functional molecule of T cells, specific TCR has been cloned from effective and specific T-cell clones and transduced into modified T cells, which may express a large quantity of cytokines or costimulating receptors to boost function of the T cells [42–47].

The EBV-associated tumor is a potential target for adoptive T-cell immunotherapy because of its latent antigen expression profile. Orentas et al. reported that, by using SAMEN retroviral vector, they could demonstrate the ability

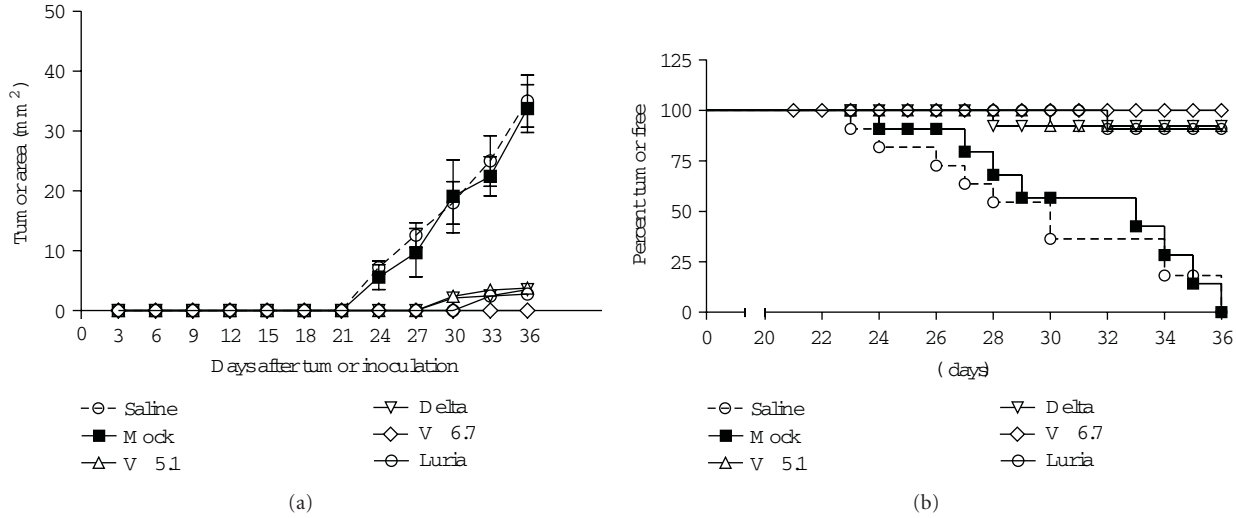

Figure 5: Significantly reduced tumor burden and enhanced tumor-free survival in nude mice implanted with LMP2-expressing tumors after infused with transduced T cells. (a) Tumor mouse model was established as described. After 7 days, mice were infused with different lentiviral transduced T cells twice at a 1-week interval. PBS-immunized mice were used as control. Tumor growth was recorded twice a week. Tumor sizes are expressed as the average of two perpendicular diameters of the tumor. Graphs show mean ± SE; $^*P < 0.05$; $n = 8$/group. (b) Comparison of survival times of tumor-bearing mice infused with T cells ex vivo transduced by lentivirus containing LMP2-TCR α and β chains driven by various T-cell promoters. Tumor burden was monitored on a weekly basis. Significant differences were observed for all transduced T-cell vaccination group. $^*P < 0.01$; $n = 14$/group. Note: tumor growth rates and survival times were discontinued when the tumor reached 1 cm^2 and the mouse was sacrificed.

to transfer CTL activity from an LMP2 peptide-specific CTL clone to a stimulated PBMC population. These TCR-transduced PBMCs showed specific immunoactivity against LMP2 targets [36]. Here, we continued this work and attempted to develop an effective lentiviral-based TCR transduction system for future clinical practice. Compared with retroviral vectors, lentiviral vectors have many advantages including the ability to transduce minimally stimulated PBMCs, and they have a potentially safer integration site preference [48, 49]. Our results showed that lentiviral vectors can effectively transduce PBMCs and $CD3^+$ cells with LMP2-specific TCRs using 4 different T-cell-specific promoters.

Using highly active T cell promoters to drive TCR α and β chains has been reported by many groups to evaluate different combinations of promoters. It has been shown to express that multiple protein subunits, viral vector, and promoters are required intensive optimization [50, 51]. We used LMP2-specific TCRs to compare 4 different promoters in lentiviral vectors. TCR α and β chains are driven by each promoter independently. Our results showed that, although lentiviral vectors of the various promoters express TCR α and β chains at different levels, all groups of transduced $CD8^+$ cells dramatically slowed or abolished the growth of LMP2-positive tumors. These results indicate that the transducing efficiency of lentiviral vectors containing different promoters does not affect the antitumor activity of CTLs. In future studies, we hope to emphasize the expansion of functional CTLs after selection rather than switching promoters to achieve higher transduction efficiency.

We have demonstrated that, for a single promoter, Vβ 6.7 is relatively superior to other promoters. Since our work solely compared T-cell-specific promoters, we could not exclude the possibility that others may have more powerful functions. Jones et al. generally compared specific and nonspecific promoters, which gave a comprehensive picture of promoter selection and combination [51]. We believe that the trend of adoptive T-cell immunotherapy is to develop safer and more effective vectors to engineer T cells. The priority is still safety. A specific T-cell promoter can limit the expression of transgenes in a relatively small subset of cells, so it is theoretically safe. Our study provides suggestions for future designing of lentiviral vectors in adoptive T-cell immunotherapy.

Conflict of Interests

The authors have contributed significantly and declare that they have no conflict of interests.

Acknowledgments

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Research Article

Detection of N-Glycolyl GM3 Ganglioside in Neuroectodermal Tumors by Immunohistochemistry: An Attractive Vaccine Target for Aggressive Pediatric Cancer

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The N-glycolylated ganglioside NeuGc-GM3 has been described in solid tumors such as breast carcinoma, nonsmall cell lung cancer, and melanoma, but is usually not detected in normal human cells. Our aim was to evaluate the presence of NeuGc-GM3 in pediatric neuroectodermal tumors by immunohistochemistry. Twenty-seven archival cases of neuroblastoma and Ewing sarcoma family of tumors (ESFT) were analyzed. Formalin-fixed, paraffin-embedded tumor samples were cut into 5 μm sections. The monoclonal antibody 14F7, a mouse IgG1 that specifically recognizes NeuGc-GM3, and a peroxidase-labeled polymer conjugated to secondary antibodies were used. Presence of NeuGc-GM3 was evident in 23 of 27 cases (85%), with an average of about 70% of positive tumor cells. Immunoreactivity was moderate to intense in most tumors, showing a diffuse cytoplasmic and membranous staining, although cases of ESFT demonstrated a fine granular cytoplasmic pattern. No significant differences were observed between neuroblastoma with and without NMYC oncogene amplification, suggesting that expression of NeuGc-GM3 is preserved in more aggressive cancers. Until now, the expression of N-glycolylated gangliosides in pediatric neuroectodermal tumors has not been investigated. The present study evidenced the expression of NeuGc-GM3 in a high proportion of neuroectodermal tumors, suggesting its potential utility as a specific target of immunotherapy.

1. Introduction

Gangliosides are a broad family of glycosphingolipids found on the outer cell membrane, initially suggested as potential targets for cancer immunotherapy based on their higher abundance in tumors when compared with the matched normal tissues [1]. They are concentrated in the nervous tissues, particularly in gray matter and synaptic junctions, although they can also be detected in most cellular types in much smaller quantities. Gangliosides are involved in cell communication and also act as regulatory elements in the immune system and in cancer progression [2, 3].

Neuroblastoma, a neoplasm originating from neural crest cells, is the most common extracranial solid tumor of childhood. Although it can arise from any site along the sympathetic nervous system, primary tumors frequently have abdominal or thoracic location [4]. Aberrant expression of NMYC oncogene plays a central role in neuroblastoma tumorigenesis, and its amplification is a major indicator of high-risk cases [5]. Most patients achieve remission with chemotherapy and surgery, but eradication of minimal residual disease (MRD) remains the major challenge in improving prognosis in high-risk neuroblastoma [4, 5].

The expression of a variety of gangliosides has been described in detail in neuroblastoma for diagnostic purposes and more recently for therapeutic targeting, since disialoganglioside (GD2)-directed immunotherapy has been reported

to improve survival in children with high-risk neuroblastoma [6]. Anti-GD2 monoclonal antibodies, as well as newer strategies such us immunocytokines and tumor vaccines, are promising approaches to eliminate resistant neuroblastoma cells [7].

The expression of N-glycolyl (NeuGc) gangliosides, and particularly the monosialoganglioside NeuGc-GM3, in neuroblastoma has not been reported to our knowledge. These gangliosides have recently received attention as a privileged target for cancer immunotherapy based on results in adult tumors [8]. Our group recently reported its expression in pediatric Wilms tumor [9]. Since this ganglioside is not detected in normal human cells, it may be considered as an interesting neoantigen for cancer immunotherapy [8,10].

The monoclonal antibody racotumomab (formerly known as 1E10), an anti-idiotype vaccine that is able to induce a specific response against NeuGc-containing gangliosides, may be considered as an option for immunotherapy in these children. Racotumomab showed promising results in clinical trials in patients with advanced breast carcinoma [11,12], melanoma, [13], and nonsmall cell lung cancer [14]. Furthermore, it was recently described that induction in cancer patients of anti-NeuGc-GM3 antibodies can cause tumor cell death by a complement-independent oncosis-like mechanism [15].

Based on this experience, we aimed to investigate the immunohistochemical expression of the N-glycolylated ganglioside NeuGc-GM3 in neuroblastoma in order to evaluate its potential as a target for immunotherapy. Cases of Ewing sarcoma family of tumors (ESFTs) were also studied, providing additional information in neuroectoderm-derived pediatric cancers.

2. Materials and Methods

2.1. Archival Cases. We retrospectively reviewed pathological specimens from 27 patients with a diagnosis of neuroblastoma or ESFT treated at the Garrahan Pediatric Hospital (Buenos Aires, Argentina). The median age of neuroblastoma patients was 22 months (range: 2 months to 11 years) at initial diagnosis. The most frequent primary site was abdominal, followed by thoracic, cervical, and axilar sites. The median age of ESFT patients was 13 years (range: 9 to 14 years), with all cases being diagnosed as primary extraosseous disease.

Neuroblastoma tumors were immunohistochemically evaluated with the monoclonal antibody NB84 (Dako Cytomation, Carpinteria, CA, USA) raised to neuroblastoma cells, while cases of ESFT were examined using an anti-CD99 antibody (Dako Cytomation). Additional immunohistochemical markers were also assessed to confirm the diagnosis of neuroblastoma or establish a differential diagnosis with other small round blue cell tumors, including synaptophysin, neurofilaments, neuron-specific enolase, Myf4, terminal deoxynucleotidetransferase, and vimentin. Patients with high-risk neuroblastoma were treated with current regimens based on evidence-based guidelines [16], thus they underwent surgical removal of the primary tumor after administration of 5 cycles of induction polychemotherapy. Children with low-risk neuroblastoma were treated with surgical resection only. Histological assessment and pathological staging were in accordance with the International Neuroblastoma Pathology Committe [17].

2.2. Fluorescent In Situ Hybridization (FISH). NMYC amplification was detected by FISH with a molecular specific DNA probe for 2p24.1 (Vysis N-MYC, Spectrum Orange Probe, Abbot Molecular, Abbot Park, IL, USA) on histological sections of neuroblastoma tumors. The number of fluorescent signals was evaluated in 200 intact, nonoverlapping nuclei of tumor cells for each probe. Positive cases were considered when the signals were of the same size and intensity. Complementary, 1p deletion was checked in neuroblastoma using a DNA probe for 1p36 (locus D1Z2). ESFT cases were analyzed for the presence of t(11;22) translocation with a probe for the EWR1 locus at 22q12.

2.3. Immunohistochemistry. The antiganglioside monoclonal antibody 14F7 was provided by the Center of Molecular Immunology (La Habana, Cuba) and used at a final concentration of 20 μg/mL. The 14F7 antibody is a mouse IgG1 that specifically recognizes the ganglioside NeuGc-GM3, as previously described [12]. Sections of 5 μm from formalin-fixed, paraffin-embedded tumor samples were used. After reaction of primary antibodies, sections were incubated with a peroxidase-labeled polymer conjugated to secondary anti-mouse antibodies using the EnVision+ System-HRP (DAB) (DakoCytomation) and developed with 3,3′-diaminobenzidine as chromogen. Proper positive and negative controls were made in every staining battery. Sections from breast carcinoma were employed as positive controls of ganglioside detection [9]. We previously demonstrated that the routine technique did not extract antigenic carbohydrate determinants of gangliosides, thus allowing immunohistochemical detection in tumor sections [18]. Detection of Ki-67 protein, a nuclear marker of proliferating cells, was performed using the specific mouse monoclonal antibody MIB-1 (DakoCytomation) at a dilution of 1 : 50.

2.4. Immunohistochemical Evaluation. NeuGc-GM3 expression was semiquantitatively evaluated, and results were scored by three independent pathologists. In the rare event of divergent evaluation, a consensus was found by discussing the cases. We graded the intensity of the staining from 0 to 3 : 0 = no staining; 1+ = mild; 2+ = moderate; 3+ = intense. Tumors were classified as negative when no staining was observed or only less than 20% of cells were positive. In addition, neuroblastoma tumors were assessed with the immunoreactive score (IRS). The percentage of NeuGc-GM3 positive cells was quantified in 5 high-power fields (in average: 2,500 cells per case) and then scored in five grades: 0 = 0–19% ; 1 = 20–39% ; 2 = 40–59% ; 3 = 60–79% ; 4 = 80–100% . IRS was calculated for each specimen by multiplication of the staining intensity and the grade of positive cells, resulting in a score ranging from 0 to 12 as described elsewhere [19].

Table 1: NeuGc-GM3 immunopositivity in neuroectodermal pediatric tumors.

Tumor variant	NeuGc-GM3		
	Positive cases[a] (%)	Positive tumor cells[b] (%)	Predominant intensity[c]
Neuroblastoma, MYCN-amplified	9/11 (81)	66 ± 11.6	2+/3+
Neuroblastoma, MYCN-nonamplified	9/11 (81)	69 ± 10.6	2+/3+
Ewing sarcoma family of tumors (ESFT)	5/5 (100)	71 ± 6.0	2+
Total	23/27 (85)	68 ± 6.3	2+

[a] Positive/total cases.
[b] Values are means ± SEM.
[c] Intensity of the positive staining was graded as 1+ = mild; 2+ = moderate; 3+ = intense.

2.5. Statistical Analysis. ANOVA followed Dunnett's test was used for multiple comparisons. Two groups were compared using two-tailed unpaired Student's test. Correlations were analyzed using the Pearson's test. A P value < 0.05 was considered statistically significant. Table and bar graph results are shown as mean ± standard error of the mean (SEM).

3. Results

Presence of NeuGc-GM3 ganglioside was evident in 23 of 27 cases (85%) of neuroectodermal tumors (Table 1), as detected by immunohistochemistry using the specific monoclonal antibody 14F7. All cases of ESFT were positive, whereas some negative cases occurred in both MYCN-amplified and -nonamplified neuroblastoma. Absence of NeuGc-GM3 expression was not associated with any particular tumor site or with the use of preoperative polychemotherapy in high-risk patients.

In average, about 70% of tumor cells were positive for NeuGc-GM3 (see also Table 1). Immunoreactivity was moderate to intense in most tumors, showing a diffuse cytoplasmic and membranous staining in neuroblastoma (Figure 1(a)), with occasional nuclear positivity as previously reported for lung tumors [20]. A fine granular cytoplasmic pattern was detected in cases of ESFT (Figure 1(b)). Interestingly, most samples of neuroblastoma with an intense NeuGc-GM3 staining corresponded to patients with an age of less than 24 months. Adjacent adrenal tissue was positive for NeuGc-GM3 in the cytoplasmic compartment in some neuroblastoma cases analyzed, suggesting shedding of gangliosides from cancer cells, as described in renal tumors [9]. No expression of NeuGc-GM3 was detected in other nontumoral tissue (Figure 1(d)).

All ESFT cases analyzed were positive for the t(11;22) translocation by FISH. No significant differences ($P > 0.05$) in NeuGc-GM3 expression were observed between MYCN-amplified and -nonamplified neuroblastoma, as assessed by the IRS (Figure 2). In the same line, no significant correlation was found between the percentage of cells positive for the Ki-67 proliferating antigen and the NeuGc-GM3 IRS ($P > 0.05$; $r = -0.1638$). As expected, tumors with MYCN amplification demonstrated a significantly higher expression ($P < 0.02$) of Ki-67 (see also Figure 2). Deletion of 1p36 was also confirmed in association with MYCN amplification in these cases. Taken together, the present data suggest that expression of NeuGc-GM3 is preserved in more aggressive neuroectodermal cancers.

4. Discussion

To the best of our knowledge, this is the first report on the expression of N-glycolylated gangliosides in pediatric neuroectodermal tumors. Our immunohistochemical study using a specific monoclonal antibody evidences NeuGc-GM3 expression in 85% of cases of neuroblastoma and ESFT. It is known that complex glycosphingolipids are abundant in cells of neuroectodermal origin [21], as well as in some epithelial cells [22]. Mammalian cells are covered by a dense glycocalyx, composed of glycolipids, glycoproteins, glycophospholipid anchors, and proteoglycans. Sialic acids attached to cell surface glycoconjugates play important roles in many physiological and pathological processes, including microbe binding that leads to infections, regulation of the immune response, and progression and spread of human malignancies [23]. The possibility that NeuGc-containing glycoconjugates are taken up directly from diet must be taken into account. However, the potential role of alternative biosynthetic pathways of NeuGc in human neoplasia, including pediatric tumors, is not known [24].

The most common sialic acids in mammals are N-acetyl (NeuAc) and NeuGc neuraminic acids. The key step in the biosynthesis of NeuGc is the conversion of NeuAc to NeuGc, which is catalyzed by the cytidine monophospho-N-acetylneuraminic acid hydroxylase [25]. NeuGc-containing gangliosides are normal components of cell membranes in all mammals except human beings. The lack of expression of NeuGc in human tissues is due to inactivation by a deletion of the hydrolase gene [26]. However, neosynthesis of carbohydrate determinants and expression of NeuGc gangliosides were observed in human cancer, possibly by diet incorporation of nonhuman sialic acid from milk or meat [10]. NeuGc-GM3 has been detected in prevalent adult cancers such as nonsmall cell lung cancer [20], breast carcinoma [27], and melanoma [28].

Ganglioside expression in ESFT has received little attention in the literature. The expression of GD2 has been reported [29] but, to our knowledge, they have not been widely used for immunotherapy [30]. Our preliminary results may be used as background for potential developments in this area. Conversely, gangliosides have been

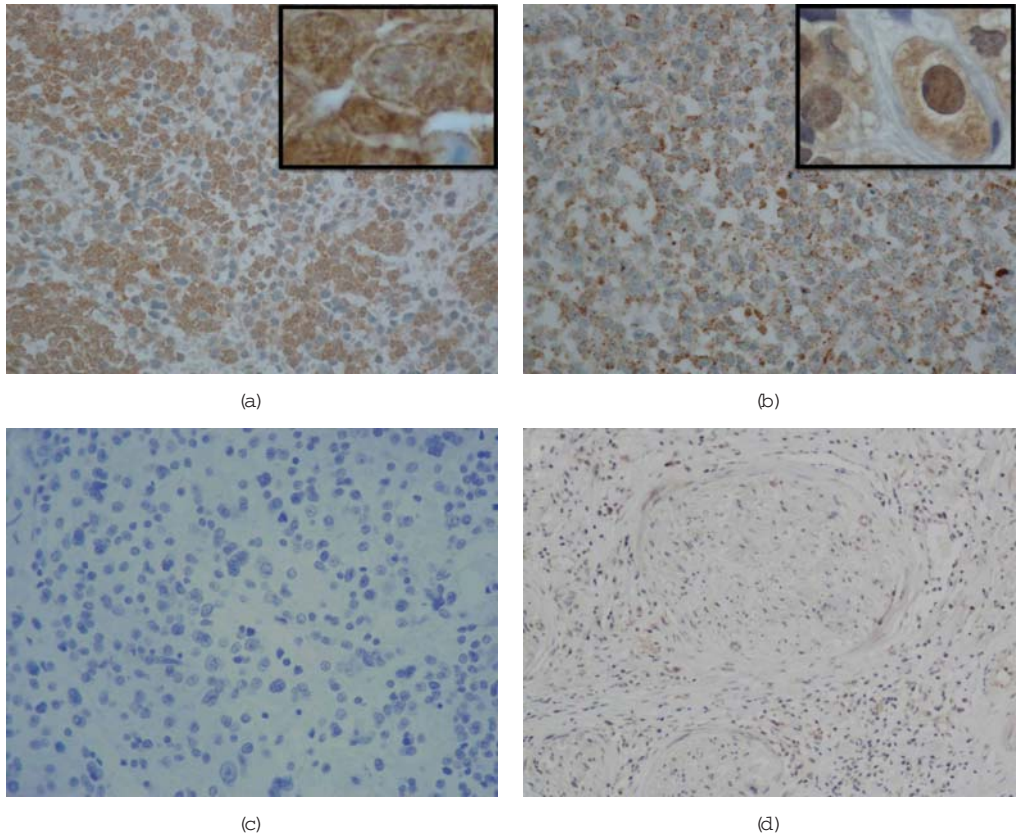

Figure 1: Immunohistochemical detection of NeuGc-GM3 ganglioside in neuroectodermal tumors. (a) Neuroblastoma (NMYC-amplified). (b) Ewing sarcoma family of tumors (ESFT). (c) Negative isotype control staining (mouse IgG1) in neuroblastoma. (d) No expression in nontumoral neural tissue. Original magnification 400X (a, b and c), 100X (d), 1,000X (insets).

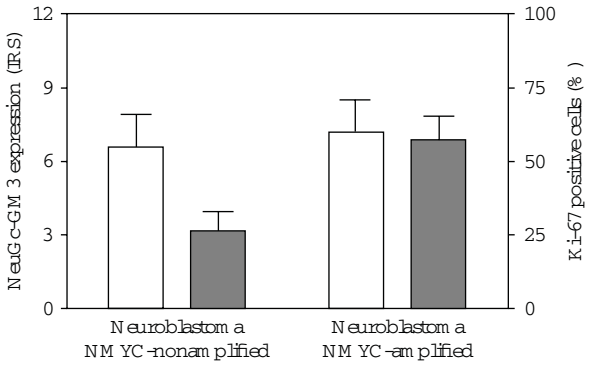

Figure 2: Expression of NeuGc-GM3 ganglioside and Ki-67 protein in NMYC-amplified and -nonamplified neuroblastoma. NeuGc-GM3 (open bars) was assessed with the immunoreactive score (IRS) and the percent of Ki-67 positive cells (closed bars) was used as a proliferation index. Data represent mean ± SEM. $P < 0.02$ (t test)

extensively studied in neuroblastoma, and a complex expression profile showing variations between neuroblastoma tumors with different malignant potential was described [31]. Moreover, patterns of ganglioside expression were used as indicators to predict patient outcome as a prognostic indicator [31].

The overexpression of GD2 has been widely reported in neuroblastoma. It is expressed in virtually all cases, and it has been used as a target for immunotherapy after the development of anti-GD2 specific antibodies. The use of anti-GD2 murine or humanized antibodies for passive immunotherapy has shown to be an effective treatment of MRD, as reported by randomized studies [6]. However, this treatment requires frequent intravenous injections, and it may be associated to severe toxicity such as hypersensitivity reactions and capillary leak syndrome appearing in up to 25% of the cases. In addition, this treatment is only available for use within clinical trials in Europe and the USA, so it is currently not an option in less developed countries. In these settings, current therapies with high dose chemotherapy and autologous stem cell rescue are available, but novel treatments for MRD are needed to improve outcome.

Based upon our present results, active immunotherapy with the anti-idiotype vaccine racotumomab, targeting NeuGc-containing gangliosides, may be feasible in children with high-risk neuroblastoma. The wide expression of NeuGc-GM3 and the favorable toxicity profile of racotumomab [11–14] may make it an attractive option for clinical use. Our results were used as background for launching a Phase I evaluation of racotumomab in children with neuroectodermal tumors in our hospital. However, these

results should be considered preliminary since a more detailed study on the expression of NeuGc-GM3 in different subsets of neuroblastoma is necessary. In addition, since expression of this gangliosides by neuroblastoma cells may be related to dietary uptake, different expression patterns in infants may be evident.

In our series, the use of preoperative chemotherapy may have changed the histopathological appearance and immunoreactivity. For instance, tumor cell subpopulations expressing NeuGc-GM3 may not be easily detectable in necrotic tumors after chemotherapy. As hypoxia-resistant cancer cells are known to have diminished response to chemotherapy, it is important to find potential target molecules for novel antitumor strategies [32]. In this context, resistant cancer cells could overexpress NeuGc-containing gangliosides under hypoxic conditions [33], but our study was not designed to assess this phenomenon. The ability of young children affected with neuroblastoma to develop an effective immune response to racotumomab vaccination may be limited, and this is also a focus of our current research.

In contrast to GD2 that is normally expressed in neural tissue of young children, NeuGc-containing gangliosides, including NeuGc-GM3, are virtually absent in normal human tissues, making these gangliosides immunogenic. In fact, antibodies that recognize NeuGc residues appear after administration of animal serum to humans [34]. Therefore, NeuGc-targeted immunotherapy may be considered an interesting candidate in neuroblastoma or other pediatric tumors such as ESFT. Although the number of cases is small, the present characterization of a specific neoantigen in neuroectodermal tumors may be of value for the design of immunotherapeutic protocols.

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Review Article

Th1 Cytokine-Secreting Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin and Prospective Use in Immunotherapy of Bladder Cancer

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Intravesical instillation of Mycobacterium bovis bacillus Calmette-Guérin (BCG) has been used for treating bladder cancer for 3 decades. However, BCG therapy is ineffective in approximately 30–40% of cases. Since evidence supports the T helper type 1 (Th1) response to be essential in BCG-induced tumor destruction, studies have focused on enhancing BCG induction of Th1 immune responses. Although BCG in combination with Th1 cytokines (e.g., interferon-) has demonstrated improved efficacy, combination therapy requires multiple applications and a large quantity of cytokines. On the other hand, genetic manipulation of BCG to secrete Th1 cytokines continues to be pursued with considerable interest. To date, a number of recombinant BCG (rBCG) strains capable of secreting functional Th1 cytokines have been developed and demonstrated to be superior to BCG. This paper discusses current rBCG research, concerns, and future directions with an intention to inspire the development of this very promising immunotherapeutic modality for bladder cancer.

1. Clinical Use of BCG in Bladder Cancer Treatment

Urothelial carcinoma of the bladder is the second most common urologic neoplasm after prostate carcinoma in the United States, with an estimated 70,530 new cases and 14,680 deaths in 2010 [1]. Global prevalence of bladder cancer is estimated at >1 million and is steadily increasing. At the time of diagnosis, 20–25% of cases are muscle invasive (stage T2 or higher) and are typically treated with surgical resection (radical cystectomy) [2]. The remainders are nonmuscle invasive bladder cancer (NMIBC) including tumors confined to the epithelial mucosa (Ta), tumors invading the lamina propria (T1), and carcinoma in situ (Tis). Transurethral resection of bladder tumor (TURBT) is the primary treatment for Ta and T1 lesions. Intravesical therapy is used as adjuvant treatment to prevent recurrence and progression of the disease after TURBT and is also the treatment of choice for carcinoma in situ. Intravesical administration of BCG, a live attenuated strain of Mycobacterium bovis widely used as a vaccine against tuberculosis, is currently the most common therapy employed for NMIBC. Since its advent in 1976 [3], BCG has been extensively used to reduce recurrence and progression of NMIBC in an attempt to preserve the bladder. BCG therapy results in 50–60% effectiveness against small residual tumors and a 70–75% complete response rate for carcinoma in situ. Adjuvant intravesical therapy was noted by the 2007 American Urological Association (AUA) panel to reduce recurrences by 24% and treatment with BCG was recommended by the panel. Unfortunately, a high percentage of patients fail initial BCG therapy and 40–50% of BCG responders develop recurrent tumors within the first 5 years [2]. In addition, up to 90% of patients experience some sort of side effects including, although rare, life-threatening complications such as sepsis.

According to the AUA's 2007 clinical practice guidelines, BCG therapy should be initiated two to three weeks following TURBT with a classic course consisting of six weekly intravesical installations. Lyophilized powder BCG (81 mg corresponding to $1–5 \times 10^8$ colony-forming units of viable mycobacteria) is reconstituted in 50 mL of saline and administered via urethral catheter into an empty bladder with

a dwell time of 2 hours. Maintenance BCG is more effective in decreasing recurrence as compared to induction therapy alone. Multiple meta-analyses support BCG maintenance and it is now firmly established in clinical practice. The European Association of Urology (EAU) and the AUA recommend one year of maintenance for high-risk patients [4, 5]. An optimal schedule/duration of therapy has yet to be determined; however, most who use maintenance follow some permutation of the Southwest Oncology Group (SWOG) program, a 3-week "mini" series given at intervals of 3, 6, 12, 18, 24, 30, and 36 months [6]. At our own institution, induction (first BCG therapy) is initiated 2 to 3 weeks following TURBT with 6 weekly installations and a 1–2 hour dwell time. For patients with carcinoma in situ, severe dysplasia, Grade 3/high grade or poorly differentiated pathology, and/or stage T1 disease, formal restaging under anesthesia is performed 6 weeks later including obtaining bilateral upper tract cytology, retrograde pyelograms, 4–5 random bladder biopsies, and prostatic urethral biopsies. If this pathology and restaging is negative, maintenance cycles may be initiated in 6 weeks. We classify three maintenance cycles A, B, and C. Maintenance A consists of 3 weekly instillations followed by cystoscopy 6 weeks later. Cytology and fluorescence in situ hybridization (FISH) in urine specimens may be obtained at this time. If cystoscopy/cytology is negative, maintenance B may be initiated 6 months after the conclusion of cycle A, again for 3 weekly treatments. Maintenance C is initiated 6 months after the conclusion of cycle B. Following cycle C, cystoscopy/cytology is repeated every 3 months for 2 years from the original diagnosis at which time it is extended to every 6 months for 1 year, and then annually.

2. Mechanism of BCG Action

Since its first therapeutic application in 1976, major efforts have been made to decipher the mechanisms through which BCG mediates antibladder cancer immunity [7, 8]. During the past decades, many details of the molecular and cellular mechanisms involved have been discovered although the exact mechanisms of BCG action still remain elusive. It is now accepted that a functional host immune system is a necessary prerequisite for successful BCG immunotherapy. It has also become clear that the effects of intravesical BCG depend on the induction of a complex inflammatory cascade event in the bladder mucosa reflecting activation of multiple types of immune cells and bladder tissue cells [7, 8]. After instillation, BCG adheres to fibronectin on the urothelial lining through a fibronectin attachment protein (FAP) on BCG [9]. This interaction between BCG and the urothelium is one of the first and most crucial steps. Attached BCG is then internalized and processed by urothelial cells including urothelial carcinoma cells (UCCs), resulting in secretion of an array of proinflammatory cytokines and chemokines such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)- , and granulocyte-macrophage colony stimulating factor (GM-CSF) [10, 11]. Following urothelial cell activation, an influx of various leukocyte types into the bladder wall occurs including neutrophils, monocytes/macrophages, lymphocytes, natural killer (NK) cells, and dendritic cells (DCs) [12–14]. These infiltrating leukocytes are activated and produce a variety of additional proinflammatory cytokines and chemokines and also form BCG-induced granuloma structures in the bladder wall [12, 14]. Subsequently, a large number of leukocyte types such as neutrophils, T cells, and macrophages are expelled into the bladder lumen and appear in patients' voided urine [15–18]. In addition, transient massive cytokines and chemokines can be detected in voided urine including IL-1 , IL-2, IL-6, IL-10, IL-12, IL-18, interferon (IFN)- , TNF- , GM-CSF, macrophage colony-stimulating factor (M-CSF), macrophage-derived chemokine (MDC), monocyte chemoattractant protein (MCP)-1, macrophage-inflammatory-protein- (MIP-) 1 , interferon-inducible protein (IP)-10, monokine induced by -interferon (MIG), and eosinophil chemoattractant activity (Eotaxin) [17, 19–24]. The urine of animals treated with intravesical BCG also showed increased IL-1 , IL-1 , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IFN- , TNF- , GM-CSF, M-CSF, and MIP-1 , regulated on activation normal T cell expressed and secreted (RANTES), and keratinocyte-derived chemokine (KC) [14]. It has been noted that the development of a predominant Th1 cytokine profile (e.g., IFN- , IL-2, and IL-12) is associated with the therapeutic effects of BCG, whereas the presence of a high level of Th2 cytokines (e.g., IL-10) is associated with BCG failure [20, 22, 23]. Thus, a shift of the cytokines produced towards a Th1 milieu is necessary for succesful BCG immunotherapy of bladder cancer. To support this, it has been observed that both IFN- and IL-12 but not IL-10 are required for local tumor surveillance in an animal model of bladder cancer [25]. Mice deficient in IL-10 genetically (IL-$10^{-/-}$) or functionally via antibody neutralization can also develop enhanced antibladder cancer immunity in response to intravesical BCG [23].

Multiple immune cell types participate in the inflammatory response induced by BCG in the bladder. It is well accepted that macrophages, an indispensable cellular component of the innate immune system, serve as the first line of defense in mycobacterial infection. Activation, maturation, and cytokine production of macrophages are primarily induced by Toll-like receptor (TLR) 2 ligation [26]. Following BCG instillation, an increased number of macrophages can be observed in bladder cancer infiltrates and the peritumoral bladder wall. Voided urine after BCG instillation also contains an increased number of macrophages and the cytokines and chemokines predominantly produced by macrophages such as TNF- , IL-6, IL-10, IL-12, and IL-18 [15, 17, 19, 22–24]. In addition to presenting BCG antigens, both human and murine macrophages are capable of functioning as tumoricidal cells toward bladder cancer cells upon activation by BCG in vitro [27–31]. The killing of bladder cancer cells by macrophages relies on direct cell-to-cell contact and release of various soluble effector factors such as cytotoxic cytokines TNF- and IFN- and apoptotic mediators such as nitric oxide (NO) [29–32]. Th1 cytokines (e.g., IFN-) enhance the induction of macrophage cytotoxicity whereas Th2 cytokines (e.g., IL-10) inhibit the induction of macrophage cytotoxicity [30, 31].

Neutrophils also compose the early responding cells to BCG instillation of the bladder and can be observed in the bladder wall and urine shortly after BCG instillation [14, 15, 17, 18]. Neutrophils are central mediators of the innate immunity in BCG infection and are activated by signalling through TLR2 and TLR4 in conjunction with the adaptor protein myeloid differentiation factor 88 (MyD88) [33]. In addition to secretion of proinflammatory cytokines and chemokines (e.g., IL-1 , IL-1 , IL-8, MIP-1 , MIP-1 , MCP-1, transforming growth factor (TGF)- , and growth-related oncogene (GRO)-) that lead to the recruitment of other immune cells [34], recent studies revealed that neutrophils are the primary source of TNF-related apoptosis-inducing ligand (TRAIL) found in the urine after BCG instillation [35, 36]. TRAIL is a member of the TNF family that induces apoptosis in malignant cells but not in normal cells. Studies have indicated that the neutrophil TRAIL response is specific to BCG stimulation rather than nonspecific immune activation. Studies have also revealed a positive correlation between urinary TRAIL level and the therapeutic effects of BCG, as BCG responders contained a significant higher amount of urinary TRAIL than BCG nonresponders [35]. These observations suggest an important role of neutrophils in BCG-induced antibladder cancer immunity. Indeed, it has been observed that depletion of neutrophils resulted in a reduced BCG-induced antibladder cancer response in a mouse model of bladder cancer [34].

Following the activation of macrophages and neutrophils in the bladder wall, driven by chemoattractants, recruitment of other immune cell types including $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and DC takes place [12, 13]. As for neutrophils and macrophages, these cell types can be found in the voided urine of patients after BCG instillation [15–17]. These effector cells produce various cytokines and chemokines to further promote BCG-induced antibladder cancer immune responses in the local milieu. In addition, DC, together with macrophages, trigger an anti-BCG-specific immune response via antigen presentation to T cells that also amplifies the BCG-induced antitumor immunity. Like neutrophils and macrophages, both T cells and NK cells are cytotoxic toward bladder cancer cells upon activation. They kill target cells via the major histocompatibility complex (MHC) restricted (e.g., for cytotoxic T lymphocytes (CTL)) and/or MHC nonrestricted pathways (e.g., for NK cells) [27, 37, 38]. Perforin-mediated lysis and apoptosis-associated killing (e.g., via Fas ligand and TRAIL) have been implicated as the major molecular effector mechanisms underlying the eradication of bladder cancer cells. These effector cell types are crucial for BCG immunotherapy of bladder cancer, as depletion of these cell types failed to develop effective antibladder cancer responses in vivo and kill bladder cancer cells in vitro [39, 40].

It has been shown that stimulation of human peripheral blood mononuclear cells (PBMCs) by viable BCG in vitro leads to the generation of a specialized cell population called BCG-activated killer (BAK) cells [41, 42]. BAK cells are a $CD3^-CD8^+CD56^+$ cell population whose cytotoxicity is MHC nonrestricted [42, 43]. BAK cells kill bladder cancer cells through the perforin-mediated lysis pathway and effectively lyse NK cell-resistant bladder cancer cells [41–43]. Macrophages and $CD4^+$ T cells have been found to be indispensable for the induction of BAK cell killing activity but have no such activity by themselves [42]. Th1 cytokines IFN- and IL-2 have also been found to be required for the induction of BAK cell cytotoxicity, as neutralizing antibodies specific to these cytokines could inhibit BCG-induced cytotoxicity [42]. BAK cells, together with lymphokine-activated killer (LAK) cells, a diverse population with NK or T-cell phenotypes that are generated by IL-2 [44, 45], have been suggested to be the major effector cells during intravesical BCG immunotherapy of bladder cancer. Other potential cytotoxic effector cells include CD1 restricted $CD8^+$ T cells [46], T cells [47], and natural killer T (NKT) cells [47, 48].

Activation of the innate immune system is a prerequisite for the BCG-induced inflammatory responses and the subsequent eradication of bladder cancer by intravesical BCG. In BCG instillation, TLRs participate in neutrophil, macrophage and DC maturation and activation. Both TLR2 and TLR4 appear to serve important but distinct roles in the induction of host immune responses to BCG or BCG cell-wall skeleton [26]. Like other microbes, BCG has surface components called pathogen-associated molecular patterns (PAMPs) that are recognized by cells of the innate immune system through TLRs during infection [49]. It is this interaction between TLRs and PAMPs that activates the cells of the innate immune system, leading to BCG-induced inflammatory responses and subsequent eradication of bladder cancer. It is known that the antitumor effect of intravesical BCG depends on its proper induction of a localized Th1 immune response. However, a systemic immune response also appears involved in intravesical BCG therapy. It has been reported that purified protein derivative (PPD) skin test often converts from negative to positive after BCG instillation and the effective treatment is associated with the development of delayed-type hypersensitivity (DTH) reaction to PPD [50]. Animal studies have also demonstrated the importance of DTH in the antitumor activity of intravesical BCG therapy [23]. Moreover, studies have shown increased levels of cytokines and chemokines in the serum (e.g., IL-2, IFN- , MCP-1, and RANTES), along with production of these cytokines and chemokines in the urine and/or bladder, during the course of BCG instillation [21, 51]. Furthermore, studies have also shown an increase in PBMC cytotoxicity against UCC after BCG instillation [21].

In addition to the ability of BCG to elicit host immune responses, evidence supports a direct effect of BCG on the biology of UCC. In vitro studies have shown that BCG is antiproliferative and even cytotoxic to UCC [27, 52] and induces UCC expression of cytokines and chemokines (e.g., IL-1 , IL-6, IL-8, TNF- , and GM-CSF) [11], antigen-presenting molecules (e.g., MHC class II, CD1 and B7-1) [53], and intercellular adhesion molecules (e.g., ICAM-1) [53]. Analysis of tumor biopsy specimens from bladder cancer patients who underwent intravesical BCG therapy further supported the ability of BCG to induce UCC expression of these molecules in vivo [13]. Moreover, the bladder urothelium of animals treated with intravesical BCG shows upregulation of HLA antigens (e.g., MHC class

I and II) and changes of many other molecules [54]. Recent studies have revealed that by cross-linking 5 1 integrin receptors, BCG exerts its direct biological e ects on UCC, including activation of the signal transduction pathways involving activator protein (AP) 1, NF B and CCAAT-enhancer-binding protein (C/EBP) [55], upregulation of gene expressions such as IL-6 and cyclin dependant kinase inhibitor p21 [55, 56], and cell cycle arrest at the G1/S transition [57]. Although some studies showed the ability of BCG to induce apoptosis in UCC [58], other studies demonstrated that BCG induced no apoptosis or even caused apoptotic resistance in UCC [59]. Further studies revealed that BCG induced UCC death in a caspase-independent manner [59] and that p21 played an important role in modulating the direct e ects of BCG on UCC [60].

3. Combination of BCG with Th1 Cytokines for Bladder Cancer Treatment

The proper induction of Th1 immunity is required for successful BCG immunotherapy of bladder cancer. Since a high percentage of patients do not respond to BCG and the e ect of BCG is associated with significant toxicity, strategies to combine BCG with recombinant (r) Th1 cytokines to enhance BCG therapeutic e cacy while reducing BCG toxicity have been employed and studied. Among Th1 cytokines, rIFN- is most extensively studied and has been shown to be safe and tolerable when used intravesically, alone or in combination with BCG, in many controlled studies [61–65]. The side-e ect profile of combination therapy is similar to BCG monotherapy including lower urinary tract symptoms such as frequency, urgency, dysuria, bladder spasm, and hematuria. Systemic fever, flu-like symptoms, and myalgias were found in <25% of patients and were self-limited. Benefits have been seen in patients with BCG failures [61–63]. Treatment with low-dose BCG (1/3 or 1/10 the standard dose) combined with rIFN- resulted in 45–53% of patients who had failed prior BCG monotherapy to remain disease-free at 24-month median followup [61, 63]. The benefit in naïve patients is currently in question with recent studies showing mixed results. A Phase III study suggested no benefit in BCG naïve patients [64]. However, no subgroup analysis was performed for carcinoma in situ or high-risk patients. Therefore, it can still be concluded that the BCG-rIFN- combination therapy may provide a benefit to patients with high-risk disease or carcinoma in situ. Data since the release of the Phase III study supports the combination therapy with BCG and rIFN- in BCG naïve patients [65]. Thus, more studies are needed to formally determine the e ect of the combination therapy for BCG naive patients. To date, a combination therapy with BCG and rIFN- 2B has been employed, particularly for patients with previous BCG failures, those with carcinoma in situ, and the elderly [63]. Optimal dose and schedule have yet to be defined in controlled trials and debate continues on the subject. At our institution, we use the standard dose of TICE BCG plus 50 million units (MU) of rIFN- 2B intravesically as induction therapy for BCG naïve patients. For BCG exposed patients, 1/3 the standard dose of BCG plus 50 MU of rIFN- 2B is utilized. The dose may be lowered for those patients experiencing lower urinary tract symptoms or low grade fever. For maintenance cycle A, we adjust the BCG dose for week 1 consisting of 1/3 the standard dose of BCG plus 50 MU of rIFN- 2B. For weeks 2 and 3, the BCG dose is lowered to 1/10 the standard dose plus 50 MU of rIFN- 2B. Maintenance cycles B and C utilize similar dosing.

Other cytokines that have been used intravesically include rIL-2, rIL-12, rIFN- , and rGM-CSF. A study demonstrated that intravesical rIL-2 was beneficial for patients with T1 papillary bladder carcinoma after TURBT showing regression of marker lesions and lack of major toxic e ects [66]. Other studies also demonstrated intravesical rIL-2 to be feasible, safe, and e ective in patients with NMIBC who were untreated or had failed prior intravesical therapy with other agents [67, 68]. A study demonstrated that intravesical rIL-12 was well tolerated by patients with recurrent NMIBC but showed no clinically relevant antitumor and immunologic e ects [69]. However, the maximum tolerated dose of rIL-12 was not reached in the study. Di erent from human studies, animal studies showed encouraging results. A survival advantage of intravesical rIL-12 was observed in a mouse orthotopic bladder cancer model [70]. Further studies for intravesical rIL-12 use are warranted. For intravesical rIFN- , a study showed the absence of major toxicity and the therapeutic e ect superior to mitomycin C for patients with NMIBC who underwent TURBT [71]. In addition, populations of leukocytes in the urothelium were significantly increased in rIFN- -treated patients confirming its induction of localized cellular immune responses. Other studies also supported the safety and antitumor activity of intravesical rIFN- monotherapy [72]. Studies also demonstrated that intravesical rGM-CSF was e ective as a prophylactic therapy for patients with NMIBC after TURBT [73, 74]. In correlation with regression of marker lesions, intravesical rGM-CSF induced leukocyte migration and activation in the bladder mucosa. Despite all these observations, however, single cytokine therapy has only been evaluated in small numbers of patients and has not yet shown compelling results in general. Indeed, in vitro studies have demonstrated that cytokines IL-2, IL-12, and TNF- , like IFN- , can enhance BCG for the induction of Th1 immune responses in human PBMC [75–77]. Thus, addition of these cytokines to BCG may provide benefits for BCG therapy particularly for BCG nonresponders or relapsers. Studies are absolutely needed to examine the combination of BCG with these cytokines for the treatment of bladder cancer.

4. Advances in Genetic Engineering of BCG for Cytokine Delivery

4.1. BCG as a Heterologous Gene Delivery Vehicle. Because of its unique characteristics, such as adjuvant potential, low toxicity, and potent immunogenicity, BCG has long been considered to be an attractive live vaccine delivery vehicle with which to deliver protective antigens of multiple pathogens. During the past 2 decades, with advances in knowledge

of mycobacterial genetics and molecular biology, a wide range of rBCG vaccine candidates expressing bacterial, viral, parasitic antigens have been developed including those for Mycobacterium tuberculosis (M.tb), human immunodeficiency virus (HIV), and hepatitis B and C viruses [78]. As early as in the 1980s, studies showed that mycobacteria were capable of delivering foreign genes that were introduced into the microbes [79, 80]. In the early 1990s, vectors carrying strong promoters from the mycobacterial major heat-shock protein genes (e.g., hsp60 and hsp70) and unique cloning sites, which allowed extrachromosomal or integrative expression of foreign antigens, were developed [81, 82]. Using these expression vectors, BCG was further demonstrated to be an effective live delivery vehicle for foreign antigens [81, 83–87]. These rBCG strains constitutively expressed foreign antigens and elicited long-lasting specific humoral and/or cellular immune responses in mice. Some of these rBCG strains even generated protective immunity against respective pathogens whose antigens were expressed by mycobacteria such as the outer surface protein A (OspA, Borrelia burgdorferi) [83], surface proteinase gp63 (Leishmania spp) [85], and surface protein A (Streptococcus pneumoniae) [86]. During that time period, vectors permitting surface expression of foreign antigens in mycobacteria or secretion from mycobacteria were also developed [83, 88]. Infection with these rBCG strains led to enhanced immune responses to some antigens in mice [83, 86, 89]. Meanwhile, vectors with various mycobacterial gene promoters, such as -antigen, P_{AN}, ag85b, 18 kDa, and furA (among many others), were also developed and demonstrated to be effective to elicit specific immune responses and/or protective immunity in different animal species including mouse, guinea pig, hamster, pig, sheep, rabbit, and monkey [78, 88, 90–92]. In addition, progress has continued in the refinement of the safety and efficacy of the rBCG vaccine vehicles. To date, numerous improved systems employed to express heterologous genes in BCG are available. Among them are vectors with limited replication or auxotrophic complementation for safe use in HIV-infected individuals, capability to replicate at a high-copy number for increased antigen delivery, dual expression cassettes for multivalent antigen delivery, capability to integrate into the genome at multiple sites for differential antigen expression, inducible elements for controlled gene expression, and expression of perfringolysin or listeriolysin (with or without urease C gene deletion) for increased $CD8^+$ T-cell stimulation. Although clinical use of rBCG vaccines is still in an early stage, studies have already demonstrated that rBCG is safe and effective in humans such as those expressing OspA and M.tb antigen 85B (Ag85B). In the years to come, more rBCG vaccines will be evaluated clinically and their usefulness in preventing human infectious dieases will become clear.

In addition to a wide range of bacterial, viral, and parasitic antigens, BCG has also been engineered to deliver tumor-associated antigens. For example, BCG expressing prostate specific molecules such as prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) have been developed. Mice immunized with the rBCG-PSA or rBCG-PSMA strain developed antigen-specific immune responses, primarily a cellular immune response [93]. We also independently developed a rBCG strain that secretes the full-length PSA. We observed that mice immunized with the rBCG-PSA strain, but not a control BCG strain carrying an empty vector, developed a potent specific CTL activity against PSA-expressing RM11psa cells (our unpublished observations). In addition, we further observed that mice primed with the rBCG-PSA strain and boosted with Ad-PSA, a replication-defective adenoviral vector carrying the full-length PSA coding sequence [94], developed enhanced PSA-specific CTL activity and IFN- expressing $CD4^+$ and $CD8^+$ T cells (our unpublished observations). Several studies including ours have also demonstrated that BCG could be engineered to express mucin-1 (MUC1), a candidate tumor-associated antigen for breast cancer and other epithelial adenocarcinomas, in a manner of multiple tandem repeats with coexpression of IL-2, GM-CSF, or CD80 [95–99]. Severe combined immunodeficient (SCID) mice reconstituted with human peripheral blood lymphocytes (PBLs) followed by immunization with these MUC1-expressing rBCG strains developed specific protective immunity against MUC1-positive human breast cancer xenografts. These observations warrant further studies in rBCG delivering tumor antigens for the treatment of malignant diseases.

Studies have shown that BCG delivery of certain biologically active molecules can induce enhanced immune responses. A study demonstrated that a rBCG strain secreting cathepsin S, a cysteine endoprotease involved in MHC class II antigen presentation, could restore intracellular cathepsin S activity and improve the capacity of BCG-infected macrophages to stimulate $CD4^+$ T cells [121]. A study also demonstrated that mice simultaneously immunized with intraperitoneal ovalbumin (OVA) and intranasal rBCG secreting the assembled pentameric cholera toxin B subunit developed a long-lasting OVA-specific mucosal IgA response as well as a systemic IgG response [122]. Remarkably, a rBCG strain expressing the genetically detoxified S1 subunit of pertussis toxin (S1PT) showed enhanced BCG adjuvant potential and, when administered intravesically, resulted in bladder weight reduction and increased survival time in a mouse syngeneic orthotopic tumor model [123, 124]. Moreover, BCG has also been engineered to express the model antigen OVA for studies of the mechanisms underlying BCG induction of antigen-specific immune responses [125]. These studies revealed that the ability of BCG to induce a delayed but persistent immune response was due to its chronicity in infection that led to a long effector phase and reduced immune cell attrition compared to Listeria monocytogenes (an acute pathogen). Furthermore, we and others have also engineered BCG to express green fluorescent protein (GFP), either alone or in combination with antigenic molecules (e.g., OVA) or cytokines (e.g., IL-2), for the studies of BCG tracking, antigen deliver, and anti mycobacterial infection [109, 126, 127].

4.2. Th1 Cytokine-Secreting rBCG. In our early studies, we developed a panel of rBCG strains that secreted mouse IL-2 or rat IL-2 under the control of the mycobacterial hsp60 promoter and -antigen signal sequence [100].

Table 1: Cytokine- and chemokine-expressing rBCG strains.

Strain	Cytokine	Species	Immunological effect	Reference
IL-2 BCG (RBD)	IL-2	m	Th1 cytprod, Antitumor, Cytotoxicity	[30, 100, 101]
IL-2 BCG (MAO)	IL-2	r	Th1 cytprod	[100]
BCG-CI	IL-2	h	Anti-BCG	[102]
BCG-CII	IL-2	h	Anti-BCG	[102]
BCG-IL-2	IL-2	m	CI, Th1 & Th2 cytprod	[103]
BCG-GM-CSF	GM-CSF	m	CI, Th1 & Th2 cytprod, DC act, Anti-M.tb	[103, 104]
BCG-IFN-	IFN-	m	CI, Th1 & Th2 cytprod, Anti-BCG	[103, 105]
rBCG/IL-2	IL-2	m	CI, Th1 cytprod, Anti-BCG	[106–108]
rBCG-IL-2/GFP	IL-2	m	CI, Th1 cytprod, Anti-BCG	[109]
rBCG (-Ag-IL-2)	IL-2	m	Th1 cytprod, Cytotoxicity	[28]
BCG-IFN-	IFN-	m	Th1 cytprod, Anti-BCG	[110]
rBCG-IFN-	IFN- 2B	h	Th1 cytprod, Cytotoxicity	[38, 111, 112]
rBCG/IL-18	IL-18	m	no clear effect	[108]
BCG IL-18	IL-18	m	Th1 & Th2 cytprod	[113, 114]
BCG-hIL2MUC1	IL-2	h	CI, Th1 cytprod, Antitumor	[95, 96]
rBCG-IFN-	IFN-	m	CI, Th1 cytprod, Antitumor	[115]
rBCG-IL-18	IL-18	m	Th1 cytprod, Anti-BCG, Cytotoxicity	[29, 30]
rBCG-huIL-2-ESAT6	IL-2	h	CI, Th1 cytprod, Cytotoxicity, HI	[116]
rBCG-IL-2	IL-2	h	Th1 cytprod	[112]
BCGMCP-3	MCP-3	m	CI, Anti-BCG	[117]
rBCG-AEI	IFN-	m	CI, HI, Anti-M.tb	[118]
rBCG-Ag85B-IL15	IL-15	m	CI, Th1 cytprod, Anti-M.tb	[119]
rBCG-MVNTR4-CSF	GM-CSF	h	CI, Th1 cytprod, Antitumor	[97, 99]
rBCG-MVNTR8-CSF	GM-CSF	h	CI, Th1 cytprod, Antitumor	[97, 99]
rBCG-Ag85B-Esat6-TNF-	TNF-	m	CI, HI	[120]

Anti-BCG: anti-BCG infection; Anti-M.tb: anti-Mycobacterium tuberculosis infection; CI: cellular immunity; DC act: dendritic cell activation; h: human; HI: humoral immunity; m: mouse; r: rat; Th1 cytprod: T helper type 1 cytokine production; Th2 cytprod: T helper type 2 cytokine production.

We demonstrated that the IL-2 secreting rBCG strains induced enhanced IFN- production by mouse splenocytes in vitro compared to wild-type BCG. Since then, numerous rBCG strains secreting different mouse and human cytokines, primarily Th1 cytokines (e.g., IL-2, IL-18, IFN- , and IFN-), have been developed (Table 1). In addition, rBCG strains secreting other cytokines or chemokines (e.g., GM-CSF, IL-15, TNF- , and MCP-3) have also emerged. Most of these cytokine- and chemokine-secreting rBCG strains showed their abilities to enhance BCG-induced cellular immune responses including Th1 cytokine production, cellular cytotoxicity, DC activation, and anti-BCG or anti-M.tb infection. Some of them even showed their antitumor effects in animal models of melanoma [101], breast cancer [96, 97, 99], and bladder cancer [115]. Certain cytokine-secreting rBCG strains also induced humoral immune responses and Th2 cytokine production other than cellular immune responses in vitro and in vivo.

5. Th1 Cytokine-Secreting rBCG in Cancer Treatment

5.1. Antitumor Studies. BCG is a potent immunoadjuvant and induces a Th1 predominant immune response that is required for effective tumor eradication in most cancer types. Genetic manipulation of BCG to secrete Th1-stimulating cytokines with simultaneous coexpression of tumor-associated antigens may therefore potentiate the induction of specific antitumor immune responses. This strategy has been approached since the emergence of cytokine-secreting rBCG strains in the 1990s. Early studies demonstrated that mouse IL-2 secreting rBCG was at least equally effective to wild-type BCG when used as an intratumoral injection or a vaccine therapy in conjunction with irradiated tumor cells in a mouse melanoma model [101]. However, it was not until recently that the potential of rBCG for treating cancer has gained further appreciation. We and others have developed rBCG strains that deliver the breast-cancer-associated antigen MUC1 in a form of multiple tandem repeats with coexpression of human IL-2 or human GM-CSF [95–97, 99]. SCID mice reconstituted with human PBL followed by immunization with the rBCG strains developed MUC1-specific cellular immune respnses and enhanced protection against MUC1-positive human breast cancer xenografts compared to control mice reconstituted with human PBL and immunized with noncytokine secreting BCG. Studies have also demonstrated that the antitumor effects of the rBCG strains were correlated with the number of MUC1 tandem repeats delivered by BCG [97, 99]. These

results suggest that these MUC1 rBCG strains coexpressing Th1-stimulating cytokines are promising candidates as breast cancer vaccines and thus deserve further investigation.

5.2. Antibladder Cancer Studies. Intravesical BCG is currently the treatment of choice for NMIBC. As for most other cancer types, the proper induction of a cellular immune response is required for successful BCG immunotherapy of bladder cancer. Studies have demonstrated that Th1 cytokine-secreting rBCG strains are capable of inducing enhanced cellular immune responses, leading to effective protection against mycobacterial infection (e.g., M. tb) and tumor progression (e.g., breast cancer) in various animal models. Unfortunately, studies on rBCG for treating bladder cancer are currently underdeveloped and, up to date, only a few reports have been available. However, studies have demonstrated that Th1 cytokine-secreting rBCG strains are superior to noncytokine secreting BCG for the induction of antibladder cancer immune responses in vitro and in vivo.

5.2.1. In Vitro Studies. It has been known that BCG stimulation of human PBMC leads to the generation of effector cells cytotoxic to bladder cancer cells in vitro [41, 42]. We recently demonstrated that stimulation of human PBMC with rBCG-IFN-, a rBCG strain secreting human IFN-2B [111], in vitro for 7 days induced enhanced PBMC cytotoxicity toward human bladder cancer cell lines T24, J82, 5637, TCCSUP, and UMUC-3 by up to 2-fold compared to control BCG carrying an empty vector [38]. This induction of enhanced PBMC cytotoxicity was correlated with increased production of IFN-, and IL-2 by rBCG-stimulated PBMC. Studies further revealed that this enhancement in PBMC cytotoxicity was dependent on BCG secreted IFN- as well as endogenously expressed IFN- and IL-2, as blockage of IFN-, IFN- or IL-2 by neutralizing antibodies during BCG stimulation reduced or abolished the induction of this enhanced PBMC cytotoxicity. Studies using NK and $CD8^+$ T cells isolated from human PBMC revealed that both cell types were responsible for the enhanced PBMC cytotoxicity induced by rBCG-IFN- with the former cell type being more predominant.

An early study demonstrated that human peripheral monocytes/macrophages were capable of functioning as tumoricidal cells toward bladder cancer UCRU-BL-17 cells upon activation by BCG in vitro [27]. It was observed that the cytotoxic activity of human monocytes/macrophages was significantly enhanced after BCG stimulation, while the naïve cells exhibited only minimum cytotoxicity. Later, more studies including ours further demonstrated that mouse macrophages could also function as tumoricidal cells toward bladder cancer cells upon activation by BCG in vitro [28–31]. Stimulation of thioglycollate-elicited peritoneal macrophages by BCG for 24 hour resulted in macrophage-mediated killing of bladder cancer MBT-2 (C3H background) and MB49 (C57BL/6 background) cells in a dose-dependent manner [30, 31]. Studies also revealed that endogenous Th1 cytokines (e.g., IL-12, IL-18, IFN-, and TNF-) played an important role in BCG-induced macrophage cytotoxicity, as blockage of these cytokines during BCG stimulation led to substantially reduced macrophage cytotoxicity toward bladder cancer cells [30]. In contrast, supplementation of BCG with Th1 cytokines (e.g., rIL-2, rIL-12, or rIL-18) increased macrophage cytotoxicity by approximately 2-fold. Consistent with these observations, rBCG strains secreting mouse IL-2 or mouse IL-18 showed enhanced macrophage-mediated killing on bladder cancer MBT-2 cells, which was correlated with increased expression of IFN-, TNF-, and IL-6 by rBCG-stimulated macrophages [30]. The effect of mouse IL-2 secreting rBCG strain on the induction of macrophage cytotoxicity toward bladder cancer MBT-2 cells was also demonstrated by a separate study [28].

5.2.2. In Vivo Studies. Although the in vitro studies have suggested the potential usefulness of Th1 cytokine-secreting rBCG strains for the treatment of bladder cancer, the effect of rBCG on treating bladder cancer in vivo has not well been studied. Up to date, only an rBCG strain secreting mouse IFN- (rBCG-IFN-) has been studied in a mouse MB49 syngeneic orthotopic tumor model [115]. This study showed that, with a low-dose treatment regimen, intravesical administration of rBCG-IFN- significantly prolonged animal survival compared to medium-treated controls, whereas BCG carrying an empty vector only slightly increased survival. In a similar experiment using the MB49 syngeneic orthotopic tumor model in IFN- knockout mice, intravesical treatment with rBCG-IFN- failed to prolong survival of mice, indicating that rBCG-derived IFN- had no measurable antitumor effect in the absence of endogenous IFN-. Studies also provided the mechanism underlying the effect of rBCG-IFN- on treating bladder cancer. As demonstrated, this rBCG-IFN- strain could specifically upregulate the expression of MHC class I molecules on MB49 cells in vitro compared to control BCG, as the MHC class I upregulation could be blocked by an inhibitory antibody to IFN-. This rBCG strain also enhanced recruitment of $CD4^+$ T cells into the bladder and further induced the local expression of IL-2 and IL-4 mRNA compared to control BCG. In addition, we have also evaluated the effects of rBCG strains secreting mouse IL-2 or mouse IP-10 (a Th1 chemokine) on treating bladder cancer in the MB49 syngeneic orthotopic tumor model and observed survival benefits of these rBCG strains (our unpublished observations). All these observations suggest that rBCG strains secreting Th1 cytokines or chemokines possess improved antitumor properties and may offer new opportunities for the treatment of bladder cancer.

Supporting Th1 cytokine-secreting rBCG, Mycobacterium smegmatis (M. smegmatis), a closely related nonpathogenic mycobacterial organism, has been engineered to secrete mouse TNF- (M. smegmatis/TNF-) and tested in a transplantable MB49 tumor model [128]. Studies demonstrated that lymphocytes from tumor-bearing mice vaccinated with M. smegmatis/TNF- produced elevated and prolonged IFN- but no IL-10 in response to mycobacterial antigen or tumor lysate stimulation in vitro. Histopathology revealed significantly increased infiltrating $CD3^+$ lymphocytes in the tumor nodules of mice receiving the recombinant

vaccine compared to those of mice receiving wild-type bacteria. These observations indicated that *M. smegmatis*/TNF- induced cell-mediated immunity. Importantly, mice implanted subcutaneously with MB49 tumor and treated at an adjacent site with the recombinant vaccine exhibited significantly reduced tumor growth with a 70% durable tumor-free survival compared to those treated with wild-type bacteria or BCG (a 10–20% long-term survival). Interestingly, treatment with *M. smegmatis*/TNF- also resulted in similar tumor growth inhibition in T-cell-deficient athymic nude mice and reduced but not abolished tumor growth inhibition in NK cell-deficient Beige mice. These observations indicated that NK-cells contribute to the antitumor effect of *M. smegmatis*/TNF- but are not solely responsible for the eradication of tumor. Like immunocompetent mice, Beige mice also developed tumor-specific memory after treatment with *M. smegmatis*/TNF- . A study also demonstrated enhanced immunotherapeutic potential of a human TNF- secreting recombinant *M. smegmatis* for treating bladder cancer [129]. The ability to deliver immunomodulatory cytokines with no pathogenic effects makes *M. smegmatis* attractive as an alternative intravesical mycobacterial agent for bladder cancer treatment.

6. Conclusion and Future View

Intravesical administration of live BCG for superficial bladder cancer is the most successful immunotherapy for solid malignancy. However, BCG therapy is associated with significant toxicity and is ineffective in approximately 30–40% of cases. During the past 2 decades, advances in mycobacterial genetics and molecular biology have offered unprecedented opportunities for the development of genetically modified BCG strains that possess improved safety profile, immunogenicity, and protective efficacy. Among these, manipulation of BCG to secrete Th1 cytokines (e.g., IL-2, IL-18, IFN- , and IFN-), alone or in combination with coexpression of bacterial or tumor antigens, represents one of the most attractive strategies for the development of improved vaccines. These types of rBCG strains have shown their potential to induce enhanced cellular immunity, leading to effective protection against mycobacterial infection (e.g., *M. tb*) and tumor progression (e.g., breast cancer) in various animal models. In bladder cancer treatment, BCG is administered intravesically; therefore, rBCG strains secreting Th1 cytokines can augment a localized cellular immune response that is crucial for effective BCG immunotherapy of bladder cancer. Since intravesical BCG in combination with local administration of Th1 cytokines such as rIFN- has already been used in humans and demonstrated to be beneficial for bladder cancer patients, Th1 cytokine-secreting rBCG strains could be very useful as improved BCG agents. Indeed, these rBCG strains have been demonstrated to be capable of inducing antibladder cancer immune responses both in vitro and in vivo in animal studies. Because of their enhanced immunogenicity, Th1 cytokine-secreting rBCG strains can be used at a lower dose, potentially reducing side effects. Further studies should focus on determination of the clinically relevant effects of rBCG strains relative to each other and optimization of rBCG dosing and treatment schedule for each rBCG strain. Application of multiple rBCG strains should be tested and development of new rBCG strains continued. Moreover, the mechanisms underlying rBCG action need to be explored. Furthermore, influence of rBCG strains on Th17 and regulatory T (Treg) cells should be evaluated as the importance of these cell types in bladder cancer has being emerged. All these efforts will afford us a better understanding of Th1 cytokine-secreting rBCG strains and the steps necessary for use of the rBCG strains for treating bladder cancer. The pace of this research must be maintained if we are to improve this gold standard therapy for bladder cancer. Th1 cytokine-secreting rBCG strains merit further appraisal as improved BCG immunotherapeutic agents for the treatment of bladder cancer.

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Review Article

Current Immunotherapeutic Approaches in Pancreatic Cancer

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Pancreatic cancer is a highly aggressive and notoriously difficult to treat. As the vast majority of patients are diagnosed at advanced stage of the disease, only a small population is curative by surgical resection. Although gemcitabine-based chemotherapy is typically offered as standard of care, most patients do not survive longer than 6 months. Thus, new therapeutic approaches are needed. Pancreatic cancer cells that develop gemcitabine resistance would still be suitable targets for immunotherapy. Therefore, one promising treatment approach may be immunotherapy that is designed to target pancreatic-cancer-associated antigens. In this paper, we detail recent work in immunotherapy and the advances in concept of combination therapy of immunotherapy and chemotherapy. We offer our perspective on how to increase the clinical efficacy of immunotherapies for pancreatic cancer.

1. Pancreatic Cancer

Pancreatic tumors usually display a ductal, an acinar, or an endocrine differentiation. The majority (approximately 95%) of pancreatic tumors arise from the exocrine component of the pancreas, and of these the significantly most common is ductal adenocarcinoma [1]. Pancreatic adenocarcinoma that is the fifth leading cause of cancer death worldwide is a lethal disease with an overall 5-year survival of only 6% [1]. Moreover, for locally advanced cancer patients, the life expectancy is about 6–8 months [1]. No adequate therapy for pancreatic cancer has yet been found, and most of patients diagnosed annually die within a year of diagnosis. Despite recent improvements in diagnostic techniques, pancreatic cancer is diagnosed at an advanced stage in most patients. Therefore, surgical resection (pancreaticoduodenectomy) can be performed in only a small number of patients [2]. Even after resection, recurrence occurs in the majority of the patients, leading to a median survival of about 18 months after resection. Although adjuvant treatment with both chemotherapy and radiation therapy was investigated, which demonstrated improvements in disease-free survival and overall survival rates [3], new therapeutic approaches are still needed.

2. Cytotoxic Chemotherapeutic Agents

Gemcitabine (2′,2′-difluorodeoxycytidine) is a chemotherapeutic drug that has become the standard treatment for advanced disease after showing superiority over 5-fluorouracil (5-FU), while chemoradiation plus systemic chemotherapy is also still widely used [4]. Therefore, gemcitabine was established as the standard first line treatment for patients with advanced disease. Gemcitabine is a nucleoside analogue that exerts its antitumor activity via multiple mechanisms of action. These include (1) incorporation of gemcitabine into replicating DNA, which inhibits DNA replication and

cell growth, (2) masked DNA chain termination, and (3) several self-potentiation mechanisms that serve to increase intracellular levels of the active compound [5]. It thus halts DNA synthesis and is invisible to DNA repair systems, leading the cells into the apoptotic pathway. However, most patients treated with gemcitabine do not survive longer than 6 months, as the tumor cells are naturally resistant to current chemotherapy. Subsequent trials aimed at improving survival have combined gemcitabine with various cytotoxic (platinums, fluoropyrimidines, or topoisomerase inhibitors) [6–10], or biological agents (tipifarnib [11], marimastat [12], or cetuximab [13]). However, the addition of the cytotoxic agents to gemcitabine did not lead to a statistically significant improvement in overall survival (OS) in patients with advanced pancreatic cancer [14–17].

3. Biological Agents

Some therapies based on mechanisms that target specific biologic pathways of tumors have commonly been referred to as "targeted therapy." While traditional cytotoxic drugs also target specific cellular process, the new generation of agents is set apart by their targeting of a pathway or molecular that derives the growth, speed, survival, or maintenance of tumor cells specially. There is a sound rationale for combining a human epidermal growth factor receptor type 1 (HER1/EGFR) inhibitor and gemcitabine in pancreatic cancer. Erlotinib (Taraceva, Genentech, South San Francisco) is a small molecule HER1/EGFR tyrosine kinase inhibitor. The human HER1/EGFR is overexpressed in many pancreatic tumors and is associated with more aggressive disease and poorer outcome [18, 19]. Blocking HER1/EGFR tyrosine kinase signaling improves the anticancer effects of gemcitabine [20]. Indeed, the combination of gemcitabine plus erlotinib significantly improved OS compared with gemcitabine alone [17]. This combination therapy first provided proof of principle of targeting HER1/EGFR in pancreatic cancer and showed erlotinib-improved survival when used concurrently with gemcitabine. Therefore, the US Food and Drug Administration (FDA) recently approved erlotinib for use in the first-line setting of advanced pancreatic cancer in combination with gemcitabine. However, this survival benefit was small, and the combination therapy increased the cost; therefore, erlotinib has not yet been widely incorporated into standard treatment protocols. Another study evaluating EGFR as a target in pancreatic cancer, using the monoclonal antibody cetuximab, has been completed. In patients with advanced pancreas cancer, cetuximab did not improve the outcome compared with patients treated with gemcitabine alone [13]. Moreover, studies evaluating monoclonal antibodies to vascular endothelial growth factor (VEGF) and using combinations of targeted agents in patients with advanced pancreatic cancer are underway.

4. Immunotherapy

The aim of antitumor immunotherapy is to induce efficient cytotoxic T lymphocyte (CTL) responses against pancreatic cancer cell. Dendritic cells (DCs) are powerful antigen-presenting cells (APCs) that play a pivotal role in the initiation, programming, and regulation of tumor-specific immune responses [21, 22]. DCs can process endogenously synthesized antigens or exogenous antigens into antigenic peptides, presented to the cell surface as MHC class I-peptide complexes, and recognized by the T cell receptor (TCR) in CD8+ T cells [23]. In contrast, exogenous antigens are captured and delivered to the compartments of the endosome/lysosome, where they are degraded to antigenic peptides by proteases and peptidases, which are complexed with MHC class II and recognized by the TCR in CD4+ T cells [23–25]. The TCR in CD8+ CTL can recognize MHC class I-peptide complexes on cancer cells and destroy cancer cells through effector molecules such as granzyme B and perforin (Figure 1) [26, 27]. Upon TCR-mediated cell activation, naive CD4+ T cells can differentiate into at least four major lineages, Th1, Th2, Th17, and regulatory T (Treg) cells all of which participate in different types of immune responses (Figure 2) [28]. The Th1 cells produce interferon (IFN)- along with proinflammatory cytokines, such as tumor necrosis factor (TNF)- and TNF- , to activate DCs, which can regulate the survival and persistence of CD8+ CTLs as memory cells [24, 29]. Th2 cells secrete interleukin (IL)-4 and IL-10 [24, 29]. The Th2 response is often associated with the humoral, antibody-based antitumor response [30, 31]. Th17 cells secrete IL-17 and IL-22, eliciting tissue inflammation implicated in autoimmunity [32–34]. There are increasing evidences that cancer cells-derived soluble factors promote the induction of tolerance through the generation of CD4+ chain of IL-2R (CD25)+ forkhead box P3 (Foxp3)+ Treg subset, which is linked to compromised antitumor immune responses [35].

The field of cancer immunotherapy is currently in an active state of preclinical and clinical investigations. The development of new treatment modalities, including specific immunotherapy, is of great importance in the treatment of pancreatic cancer. In support of the immunotherapy approach are the findings that pancreatic cancer cells express TAAs such as Wilms' tumor gene 1 (WT1) (75%) [36], mucin 1 (MUC1) (over 85%) [37], human telomerase reverse transcriptase (hTERT) (88%) [38], mutated K-RAS (73%) [38, 39], survivin (77%) [40], carcinoembryonic antigen (CEA) (over 90%) [41], HER-2/neu (61.2%) [42], or p53 (67%) [43] as potential targets for immunotherapy. Immunotherapies aim to recruit and activate T cells that recognize TAAs-specific antigens. Moreover, pancreatic cancer cells themselves actively contribute to immune suppression through production of immune suppressive cytokines (e.g., TGF- , IL-10, and IL-6) and by expressing surface molecules that mediate immune suppression (e.g., vascular endothelial growth factor (VEGF), Fas ligand (Fas-L), programmed death-1 ligand (PD-L1) and indolamine-2, and 3-dioxygenase (IDO)) [44]. In addition, the environment in pancreatic cancer is consisted of not only cancer cells but also immune suppressive cells such as cancer-associated fibroblasts (CAFs), tolerogenic DCs, myeloid-derived suppressor cells (MDSCs), immunosuppressive tumor-associated macrophages (TAMs), and Treg cells [44] (Figure 3). As a result,

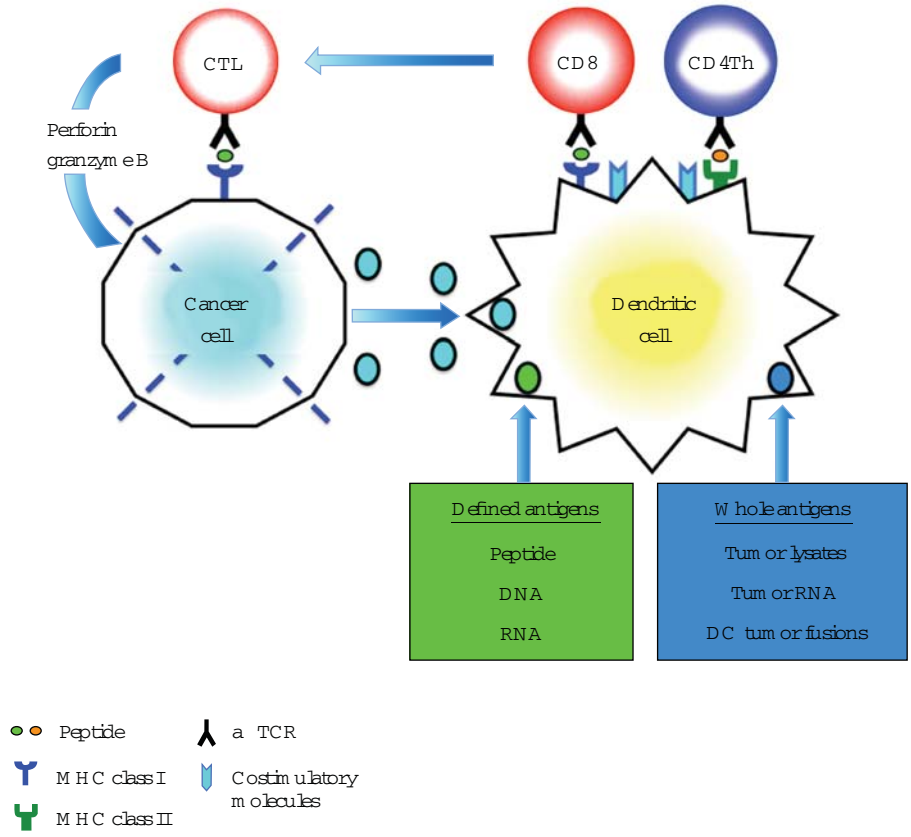

Figure 1: CTL induction by DCs. Antigens are taken up and degraded into peptide fragments by antigen-presenting cells, such as immature DCs. DCs process tumor-derived peptides and MHC class I peptides derived from DCs. They form MHC class I-peptide complexes, in the endoplasmic reticulum, which are transported to the surface of DCs and presented to CD8+ T cells. DCs also synthesize MHC class II peptides in the endoplasmic reticulum, which are transported to the cytoplasm where MHC class II-peptide complexes are assembled with tumor-derived peptides and presented to CD4+ T cells. The CD4+ T cells produce increased amounts of IL-2, which drives CD8+ T-cell proliferation. CD8+ T cells then become CTL, which can destroy cancer cells through effector molecules such as granzyme B and perforin.

immunosuppressive cells inhibit antitumor immunity by various mechanisms, including depletion of arginine and elaboration of reactive oxygen species (ROS) and nitrogen oxide (NO) [44]. Finally, an immunosuppressive tumor microenvironment induced by pancreatic cancers suppresses CD8+ CTL function through secretion of IL-10 and TGF- from Treg cells [45, 46]. The accumulation of these immune suppressive cells in pancreatic cancer might be closely related to the extent of disease and correlated well with disease stage. Therefore, immunotherapies that struggle against pancreatic cancer cells with antigen-specific CTLs as well as depletion of Treg cells may tip the balance in favor of immunostimulation. Currently, the field of cancer immunotherapy using peptide- or cell- (DC or whole tumor cell)-based approaches is in an active state of preclinical and clinical investigations.

5. Peptide Vaccines

TCR engagement by peptide/MHC constitutes the main signal for the activation of naive CD4+ and CD8+ T cells. Although CD8+ naive T cells recognize peptides derived from TAAs bound by MHC class I molecules, it is not sufficient to initiate a productive generation of antigen-specific CTLs. Full induction of CTLs requires additional signals driven by costimulatory molecules on DCs. CD8+ CTLs can respond to TAAs-derived peptides presented in the context of MHC class I molecules on tumor cells. Therefore, many investigators have tried to identify MHC class I-binding peptides that could be utilized to develop tumor vaccines for treatment of cancer patients. Peptide-based cancer vaccines are preparations made from antigenic protein fragments (called epitopes) that represent the minimal immunogenic region of antigens [47, 48]. The increased understanding of antigen recognition at molecular level has resulted in the development of rationally designed peptide vaccines. Indeed, the peptide-based cancer vaccines for pancreatic cancer have undergone phase I/II clinical trials [49, 50]. The major advantages of peptide vaccines are that they are simple, safe, stable, and economical. Induction of CTLs need peptides derived from TAAs to be presented on the surface of APCs such as DCs in the context of HLA molecules. However, several obstacles limit the widespread usefulness of peptide vaccines. The drawback of this strategy comes from numerous factors: (i) a limited number of known synthesized

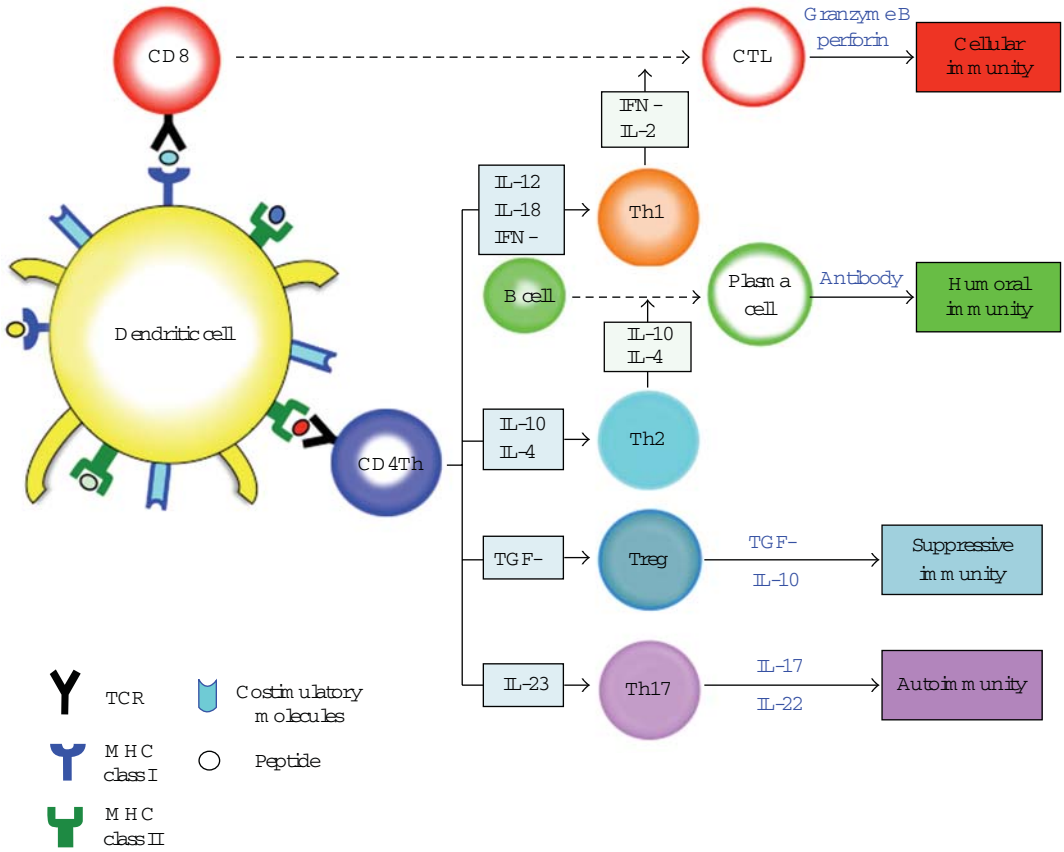

Figure 2: Immune homeostasis. Upon TCR-mediated cell activation, naive CD4 T cells can differentiate into four major lineages, Th1, Th2, Th17, and Treg cells that participate in different types of immune responses. The Th1 cells produce IFN- and IL-2, resulting in induction of CD8+ CTLs. Th2 cells secrete IL-4 and IL-10. The Th2 response is associated with the humoral, antibody-based antitumor response. Th17 cells secrete IL-17 and IL-22, eliciting tissue inflammation implicated in autoimmunity. Treg cells that secrete TGF- and IL-10 suppress effector Th1 or Th2 cells.

short peptides cannot be available in many HLA molecules [51–53], (ii) CD8+ CTLs may be ineffective in reacting with pancreatic cancer cells down-regulated by certain tumor antigens and MHC class I molecules, which may appear during the course of tumor progression [22], (iii) impaired function of APCs in patients with advanced pancreatic cancer [54, 55], and (iv) MDSC or Treg cells in tumor environment produce immunosuppressive cytokines such as IL-10 and TGF- [26].

Vaccination with synthetic peptides, particularly MHC class I-binding epitopes, has been performed in pancreatic cancer (Table 1). In a phase I/II trials, vaccination for the patients with advanced pancreatic cancer using mutant K-ras [39, 56, 57], MUC1 [58, 59], or telomerase [60] peptides was significantly associated with immune responses. Gjertsen et al. [56] first reported mutant K-ras peptide vaccines for pancreatic cancer. Since native epitopes have relatively low immunogenicity, granulocyte-macrophage colony-stimulating factor (GM-CSF) was applied to achieve efficient vaccination in the study. Among 48 patients with pancreatic cancer (10 surgically resected and 38 with advanced disease), vaccination of mutant K-ras peptides in combination with GM-CSF resulted in immune responses and prolonged survival. Moreover, another group also reported that vaccination of 24 patients with resected pancreatic cancer with K-ras peptide in combination with GM-CSF proved to be safe without tumor regression [57]. In an MUC1 peptide vaccine, vaccination of 16 patients with resected or locally advanced pancreatic cancer with 100 mer MUC1 peptide and SBAS2 adjuvant resulted in low but detectable MUC1-specific immune responses in some patients [59]. Moreover, vaccination with 100 mer MUC1 peptide and incomplete Freund's adjuvant resulted in increased circulating anti-MUC1 IgG antibody in some patients [58]. In addition, augmented immune responses and prolonged survival were observed following vaccination of advanced pancreatic cancer patients with telomerase peptide and GM-CSF [60]. Recent protocols using personalized peptides demonstrated frequent induction of tumor reactive T cells [61]. In this regimen, prevaccination peripheral blood mononuclear cells (PBMCs) were screened for their reactivity in vitro to each peptide in patients, and only the reactive peptides were vaccinated to 11 patients with advanced pancreatic cancer. In the personalized peptide vaccines, augmented immune responses to at least one of peptides used for vaccination were observed in

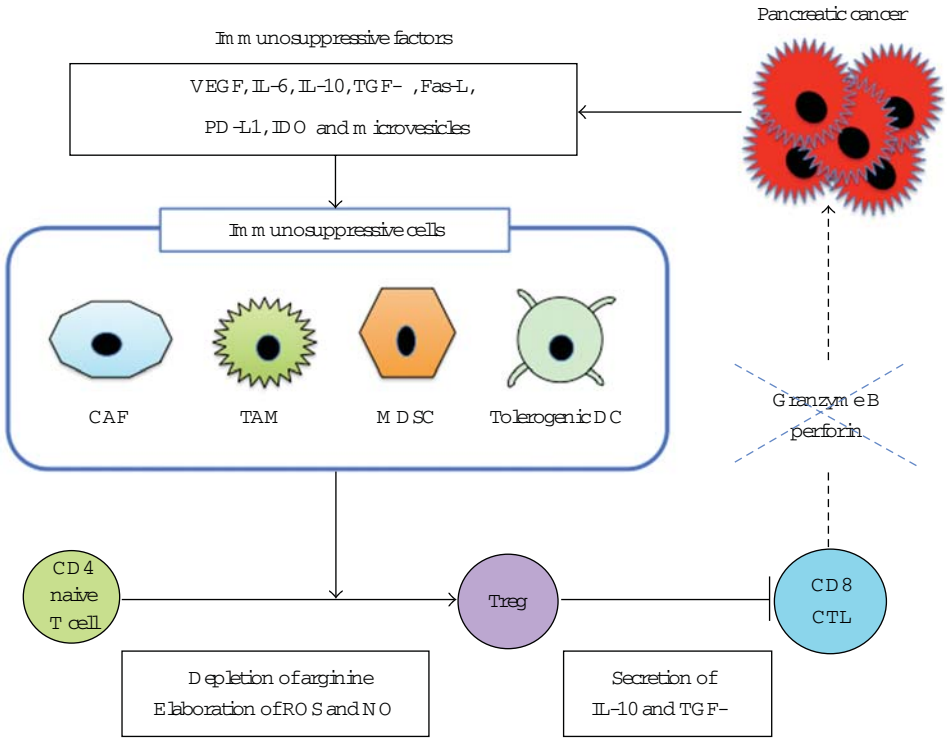

Figure 3: Pancreatic cancers induce an immunosuppressive tumor microenvironment. Pancreatic cancer cells secrete various immunosuppressive factors such as VEGF, IL-6, IL-10, TGF- , Fas-L, IDO, PD-L1, and microvesicles, all of which promote the accumulation of TAM , MDSC, or tolerogenic DC. These immunosuppressive cells inhibit antitumor immunity by various mechanisms, including depletion of arginine and elaboration of ROS and NO. An immunosuppressive tumor microenvironment induced by pancreatic cancers suppresses CD8+ CTL function through secretion of IL-10 and TGF- from Treg cells. All contribute to pancreatic cancer-induced immune suppression.

the postvaccination PBMCs [62]. In these all peptide vaccines, only a limited success has occurred in clinical trials. The short peptide can be loaded exogenously in MHC class I molecules and presented by DCs within a few days after injection to the patients. Moreover, the short peptide vaccines are not immunogenic enough. The more attractive peptide-based vaccines may be synthetic long peptides to induce antigen-specific polyclonal CD4+ and CD8+ T cells [63]. As long synthetic peptides are not able to bind directly on MHC class I or II molecules on DCs, they need to be taken up, processed, and presented by DCs. The long peptide vaccines can present MHC class I- and II-restricted epitopes long time, thus eliciting both CD4- and CD8-mediated immune recognition [64]. Peptide vaccines aimed at the treatment of established cancer may require long-lived presentation of epitopes by MHC class I and II molecules on appropriately activated DCs. Such presentation is essential for induction of robust therapeutic T-cell responses.

In a phase I study using long synthetic mutant ras peptides, Weden et al. [65] treated 23 patients who were vaccinated after surgical resection for pancreatic cancer. Long-term immunological memory responses to the vaccines were observed. Strikingly, 10-year survival was 20% (four patients out of 20 evaluable) versus zero (0/87) in a cohort of nonvaccinated patient treated in the same period. Cancer vaccines for pancreatic cancer may be tested to prevent recurrence and metastasis after surgical resection. Furthermore, peptide vaccines to boost immune responses in combination with chemotherapy to overcome robust cancers may be the key elements for the development of therapeutic cancer vaccines. Indeed, Wobser et al. [40] reported a case of complete remission (CR) of liver metastasis of pancreatic cancer refractory to gemcitabine chemotherapy under vaccination with a survivin peptide.

6. Whole Tumor Cell Vaccines

Despite the identification of peptides, autologous whole tumor cells remain a potent vehicle for generating antitumor immunity. This is because tumor cells express all relevant candidate TAAs, including both known and unidentified. In the clinical setting, an autologous whole tumor cell vaccine depends on the availability of adequate numbers of tumor cells. As only 10–15% of pancreatic cancer patients diagnosed are eligible for surgical, autologous pancreatic cancer cells may not be provided in most of the patients. Moreover, even if the patients are treated by surgical resection, it is difficult to prepare sufficient numbers of tumor cells due to the length of culture time and potential contamination of bacteria and fungus [55, 66]. To circumvent this problem,

Table 1: Peptide vaccines.

Patients	Peptide vaccine	Adjuvant	Response	Ref.
10 resected and 38 advanced pancreatic caner	Mutant K-ras peptide	GM-CSF	Immune response to the peptide vaccine showed prolonged survival compared to nonresponders. K-ras-specific T cells were selectively accumulated in the tumor.	[56]
24 resected pancreatic cancer	Mutant K-ras peptide	GM-CSF	No elicitable immunogenicity and unproven efficacy was observed.	[57]
16 resected or locally advanced pancreatic cancer	100 mer MUC1 peptide	SB-AS2 adjuvant	Detectable MUC1-specific humoral and T-cell responses were detected in some patients.	[59]
6 advanced pancreatic cancer	100 mer MUC1 peptide	Incomplete Freund's adjuvant	One patient showed a tendency for increased circulating anti-MUC1 IgG antibody.	[58]
48 advanced pancreatic cancer	Telomerase peptide	GM-CSF	Immune responses were observed in 24 of 38 evaluable patients. One-year survival for the evaluable patients in the intermediate dose group was 25%.	[60]
11 advanced pancreatic cance	Personalized peptide vaccine		The 6- and 12-month survival rates for patients who received > 3 vaccinations (n = 10) were 80% and 20%, respectively.	[62]
23 resected pancreatic cancer	Mutant ras long peptide		Seventeen of 20 evaluable patients (85%) responded immunologically to the vaccine. Ten-year survival was 20% (four patients out of 20 evaluable).	[65]
1 liver metastasis of pancreatic cancer refractory to gemcitabine	Survivin peptide		The patient initially underwent partial remission of liver metastasis which proceeded after 6 months into a complete remission with a duration of 8 months.	[40]

allogeneic tumor cell lines may be used instead of autologous tumor cells [66]. This strategy has numerous advantages: (i) allogeneic tumor cell lines are well characterized as TAA source, (ii) specific TAAs do not need to be identified for vaccination, (iii) allogeneic tumor cell lines, which shared with TAAs, can grow well in vitro; thus, there is no limiting factor for preparation of tumor cells, (iv) it is not necessary to determine HLA typing of patients and allogeneic tumor cells, because autologous DCs can process and present multiple TAAs from allogeneic tumor cells owing to cross-presentation in the context of appropriate MHC class I and II alleles, and (v) polyclonal antigen-specific CD4+ and CD8+ T cells can be generated, which may protect against tumor escape variants. While currently explored allogeneic approaches in whole tumor cell-based vaccination procedures represent an improvement in terms of standardization over their autologous counterparts, they nevertheless entail the culture of large batches of cells under good manufacturing practice (GMP) grade conditions. One of major challenges to develop an allogeneic tumor cell-based vaccine strategy is to overcome the potential hazards of fetal calf serum (FCS) that limit safety in clinical trials [55]. Optimization of these in vitro culture methodologies is required.

In clinical trials, allogeneic whole tumor cells (melanoma, prostate, and pancreatic cancer), transduced with GM-CSF, have been applied clinically and shown to induce antitumor immunity [67–69]. In this trial, whole allogeneic tumor cells were genetically modified to secrete the immune stimulatory cytokine, GM-CSF, and then irradiated to prevent further cell division. GM-CSF is now recognized to be the crucial growth and differentiation factor for DCs. Therefore, this approach is based on the concept that GM-CSF is required at the site of the tumor to effectively prime TAAs-specific immunity. Allogeneic GM-CSF-secreting pancreatic cancer vaccine was conducted (Table 2). The vaccines induced systemic antitumor immunity against autologous pancreatic cancer cells [67]. The same group [70] administrated GM-CSF-secreting allogeneic pancreatic cancer cells in sequence with cyclophosphamide in patients with advanced pancreatic cancer. The approach showed minimal treatment-related toxicity and mesothelin-specific T-cell responses. Moreover, combination of the vaccine and cyclophosphamide resulted in median survival in a gemcitabine-resistant population similar to chemotherapy alone. It was also reported that combination of the vaccines and chemoradiation demonstrated an overall survival that compares favorably with published data for resected pancreas cancer [69].

7. DC-Based Vaccines

DCs derive their potency from the prominent expression of MHC class I and II, costimulatory (CD80 and CD86), and adhesion molecules that provide secondary signals for the activation of naive CD4+ and CD8+ T cells [24]. Therefore,

Table 2: Whole tumor cell-based vaccines.

Patients	Whole tumor cell-based vaccines	Combination	Response	Ref.
14 resected pancreatic cancer	Allogeneic GM-CSF-secreting pancreatic cancer cell		Vaccination induced increased delayed-type hypersensitivity (DTH) responses to autologous tumor cells in three patients. 3 patients also seemed to have had an increased disease-free survival time, remaining disease-free at least 25 months after diagnosis.	[67]
30 advanced pancreatic cancer	Allogeneic GM-CSF-secreting pancreatic cancer cell	Vaccine alone or in sequence with cyclophosphamide	CD8+ T-cell responses to HLA class I-restricted mesothelin epitopes were identified predominantly in patients treated with cyclophosphamide and immunotherapy. Cyclophosphamide-modulated immunotherapy resulted in median survival in a gemcitabine-resistant population similar to chemotherapy alone.	[70]

a major area of investigation in cancer immunotherapy involves the design of DCs-based cancer vaccines [71, 72]. Several strategies to deliver TAAs including defined or whole antigens to DCs have been developed to generate a potent CTL response against tumor cells in murine and human systems (Figure 4). DCs have been pulsed with synthetic peptide derived from the known tumor antigens [73], tumor cell lysates [74], apoptotic tumor cells [75], or RNA derived from tumor antigens [76] and transfected with whole tumor cell DNA [77] or RNA [78]. Moreover, DCs have been fused with tumor cells to induce antigen-specific polyclonal CTL responses [79]. In the DC/tumor cell fusion approach, whole TAAs including those known and those yet unidentified are processed endogenously and presented by MHC class I and II pathways in the context of costimulatory signals [80–82]. Although DC-based vaccines have proven clinically safe and efficient to induce tumor-specific immune responses, only a limited number of objective clinical responses have been reported in cancer patients [83–86]. These relatively disappointing results have prompted the evaluation of multiple approaches to improve the efficacy of DC-based vaccines.

DC-based vaccines have also been used for pancreatic cancer (Table 3). The human tumor antigen mucin, encoded by the gene MUC1, is a high-molecular-weight glycoprotein that is overexpressed in adenocarcinomas including pancreatic cancer and hematological cancers and can be recognized by cytotoxic T lymphocytes (CTLs) and monoclonal antibodies [87]. A vaccine consisting of liposomal MUC1-transfected autologous DCs was evaluated in a clinical phase I/II trial. In MUC1 peptide-loaded DC vaccines in pancreatic and biliary cancer patients following resection of their primary tumors, 4 of the 12 patients followed for over four years were alive, all without evidence of recurrence [88]. Moreover, MUC1-specific immune responses were also observed even in patients with pretreated and advanced disease, following immunization with DCs transfected with MUC1 cDNA [89]. As hTERT is the catalytic subunit of telomerase and a prototype for a novel class of universal tumor antigens, it is one of widely applicable targets recognized by CTLs [90]. In the human system, DCs transfected with hTERT mRNA have previously been shown to induce CTL responses to hTERT in vitro [91]. Furthermore, findings from initial clinical trials demonstrate that hTERT-specific immune responses can be safely induced in cancer patients [92]. A patient who could not continue chemotherapy due to severe neutropenia had been treated with autologous DCs transfected with hTERT mRNA for 3 years and resulted in no evidence of active disease. Moreover, the complete remission (CR) was associated with induction of hTERT-specific CD4+ and CD8+ T cells [93].

8. DNA Vaccines

Cell-based cancer vaccines cause antitumor immune response at first. But they become less effective over time because the induced immune system recognizes them as foreign and quickly destroys them. DNA vaccines that consist of TAAs and additional immune-stimulatory factors have an advantage over cell-based vaccines because it can provide prolonged antigen expression, leading to amplification of immune responses and inducing memory responses against weakly immunogenic TAAs. Moreover, as DNA might be taken up by cells and the encoded antigen is processed through both endogenous and exogenous pathways, DNA vaccines that administered via intramuscular injection allow for an immune response to multiple potential epitopes within an antigen to be generated regardless of the patient's MHC profile [95]. DNA vaccines are now being studied in clinical trials for melanoma and prostate cancer. In pancreatic cancer, DNA vaccination targeting MUC1 [96] or survivin [97] has been studied in murine models and resulted in antitumor immune responses.

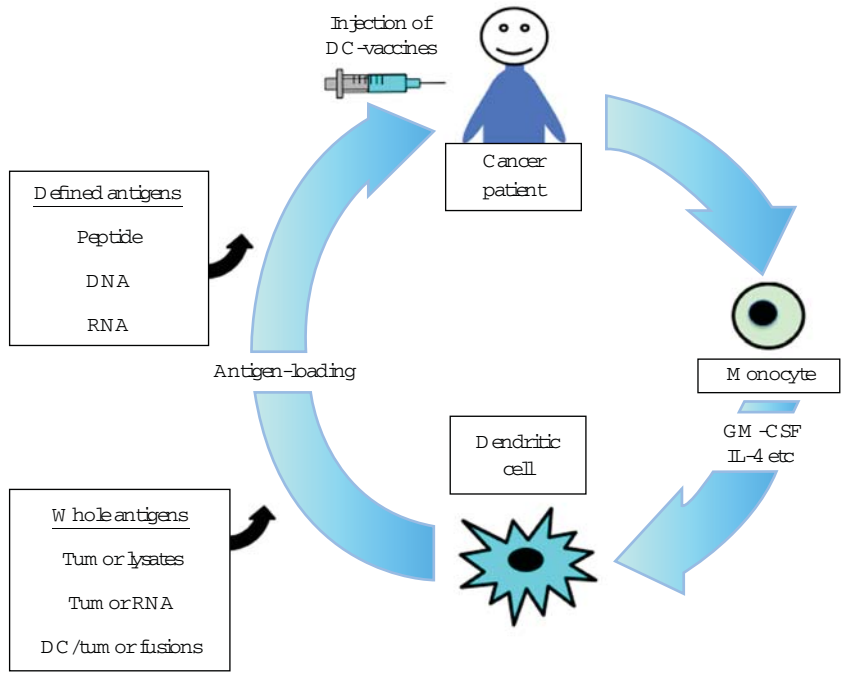

Figure 4: Strategies to deliver defined or whole antigens to DCs. DCs used for cancer vaccines have been generated from the peripheral blood monocytes of the patients using cytokines including GM-CSF and IL-4. To generate antigen-specific CTL response against tumor cells, DCs have been loaded with defined or whole tumor-associated antigens. For example, DCs loaded with synthetic peptide, antigenic DNA, or RNA have been used. Moreover, whole tumor-associated antigens including defined and unidentified have been also loaded to DCs.

Table 3: DC-based vaccines.

Patients	DC-based vaccines	Response	Ref
12 pancreatic and biliary cancer patients with resected tumors	MUC1 peptide-loaded DC	4 of the 12 patients followed for over four years were alive.	[88]
10 patients with advanced breast, pancreatic, or papillary cancer	DC transfected with MUC1 cDNA	A vaccine-specific delayed-type hypersensitivity (DTH) reaction was observed in 3 out of 10 patients. 4 patients showed a 2- to 10-fold increase in the frequency of MUC1-specific IFN-gamma-secreting CD8+ T cells.	[89]
1 patient who could not continue chemotherapy due to severe neutropenia	DC transfected with hTERT mRNA	The patient showed no evidence of active disease based on PET/CT scans. The patient developed an immune response against several hTERT-derived Th and CTL epitopes.	[93]
49 patients with advanced pancreatic cancer refractory to standard chemotherapy	Peptide (WT1, MUC1, CEA, and CA125)-loaded DC Gemcitabine/S-1	2 patients showed complete remission (CR), 5 partial remission (PR) and 10 stable disease (SD). Median survival time was 360 days.	[94]

9. Chemotherapy and Immunotherapy

Recently, new paradigms have emerged in the field of cancer vaccine research. In particular, the potential use of combination therapies that incorporate immune modulators and standard radio- and chemotherapy to synergize with cancer vaccines has been discussed. Cytotoxic chemotherapy is generally considered immunosuppressive, because of its toxicity for dividing cells in the bone marrow and peripheral lymphoid tissue. Therefore, the combination of cancer vaccines with chemotherapies has been considered to be inappropriate because the immunosuppressive effects of the chemotherapy would negate the efficacy of cancer vaccines. However, increasing evidences have been mounting to suggest that immunotherapy has the possibility of achieving better success when used in combination with conventional

chemotherapy [98, 99]. A standard cytotoxic agent, gemcitabine, not only exerts direct antitumor activity, but also mediates immunological effects relevant for cancer immunotherapy [100–102]. Cross-presentation of TAAs by DCs is essential for induction of augment CTL responses. Treatment of cancer cells and DCs with gemcitabine results in enhanced cross-presentation of TAAs by DCs, CTL expansion, and infiltration of the tumor, all of which are associated with augmented CTL [103–106]. The increase in cross-presentation did not lead to tolerance [104, 105]. Moreover, gemcitabine reduced the number of myeloid suppressor cells but did not reduce CD4+ T cells, CD8+ T cells, NK cells, macrophages, or B cells [107]. Therefore, gemcitabine may be not immunosuppressive and enhance responses to immunotherapy administered to activate or support immune responses directed toward driving effector immunity to pancreatic cancer cells [108]. Indeed, combination of DCs pulsed tumor cells with gemcitabine augmented therapeutic efficacy in vivo in a murine pancreatic cancer model [109]. Moreover, Ramakrishnan et al. [110] have reported that chemotherapeutic agents caused upregulation of cation-independent mannose 6-phosphate receptor (CI-MPR) expression on cancer cells and a concurrent increase in the uptake of granzyme B by activated CTLs in a large number of neighboring cancer cells. As a result, CTLs may cause apoptosis in large numbers of cancer cells manifesting in a clinically evident antitumor effect. Thus, such a combination therapy may be very promising approach to the treatment of patients with advanced pancreatic cancers.

Tumors that develop drug resistance would still be a suitable target for immunotherapy [111]. It has been well known that the majority of patients with advanced pancreatic cancer that respond initially to standard chemotherapies ultimately undergo relapse due to the survival of small populations of cells with cancer-initiating/cancer stem cell (CSC) fraction [112]. These CSCs are a subpopulation of the tumor more capable than other cells to self-propagate, initiate new tumors differentiate into bulk tumor, and therefore sustain tumor growth. Although chemotherapy kills most cancer cells, it is believed to leave CSCs behind, which might be an important mechanism of resistance [113]. CSCs are resistant to a variety of treatments, including chemotherapy and radiotherapy, with varied mechanisms of resistance, including high expression of ATP-binding cassette (ABC) drug transporters, an active DNA-repair capacity, and a resistance to apoptosis [113, 114]. Recently, CSCs have been isolated from various types of malignancies, including pancreatic cancer [114–118]. According to the manner of expression in CSCs, TAAs can be classified into two categories: (i) CSC-specific antigens, such as SOX2 [119] and ALDH1A1 [120] and (ii) shared antigens, such as CEP55 [121], MUC1 [122], or WT1 [123, 124] between CSCs and more differentiated subpopulations. Several methods to isolate CSC have been reported, including cell surface markers such as CD44, CD24, CD133, or epithelial-specific antigen (ESA) and side population (SP) cells using Hoechst 33342 dye [115, 119, 120]. Purified tumor stem cells from a patient can be used to immunize the patient or to activate the donor's immune cells against the tumor stem cells [113]. Therefore, the development of strategies that target the CSC population by immunotherapy may be highly desirable. Success of these potential therapies will depend on how well immunological responses to CSCs can be modulated by vaccines. We recently generated hybrid cells by fusing DCs and CSCs to activate potent CSC-specific CTL responses. The DC/CSC fusions induced proliferation of T cells with high expression levels of IFN- and enhanced killing of CSCs in vitro [111]. Moreover, peptide-based cancer vaccines or adoptive cell transfer of the CSC-specific CTL clone is a possible approach for targeting chemotherapy-resistant CSCs [120]. These findings open a novel field of investigations for future clinical trial design, taking into account the immunostimulatory capacity of chemotherapy such as gemcitabine, and using them in combined chemoimmunotherapy strategies in patients with pancreatic cancer [103, 104, 106, 125, 126]. Moreover, it seems that a period of time exists between the start of chemotherapy and immunotherapy. As the fact that even without chemotherapy, antitumor immune responses induced by immunotherapy cannot be sustained for a long period of time in patients with cancer. It would be important to establish the optimum timing and scheduling of immunotherapy and chemotherapy, to identify whether this synergistic effect is limited to a specific type of chemotherapy and whether immunotherapy can also augment the clinical effect of chemotherapy [44, 110, 127]. A combined approach of conventional therapies such as radiation or chemotherapy kills the bulk of tumor cells, and CSC-reactive CTL that target CSC fraction may represent a more promising approach for the treatment of patients with advanced pancreatic cancer (Figure 5).

In clinical trials, patients with advanced pancreatic cancer had been treated by combination therapy of standard cytotoxic agent, gemcitabine with personalized peptides [49, 128], or vascular endothelial growth factor receptor 2 (VEGFR2) [50]. The reactive personalized peptides (maximum of 4 kinds of peptides) were administered with gemcitabine to patients with nonresectable pancreatic cancer. Median survival time of all 21 patients was 9.0 months with a one-year survival rate of 38%. Immune boosting in both cellular and humoral responses was well correlated with overall survival. Moreover, in combination therapy of peptide for VEGFR2 with gemcitabine for patients with metastatic and unresectable pancreatic cancer, the median overall survival time of all 18 patients who completed at least one course of the treatment was 8.7 months. VEGFR2-specific CTL responses could be induced by the combination therapy. The survival benefit of combination therapy of peptide vaccines and gemcitabine in comparison with gemcitabine alone needs to be confirmed in randomized clinical trials. Similar findings are also observed in combination therapy of DCs-based cancer vaccines and gemcitabine. Five patients with locally advanced pancreatic cancer had been treated with gemcitabine, OK-432-stimulated DCs injected into the tumor sites, and intravenous infusion of lymphocyte-activated killer cells stimulated with anti-CD3 monoclonal antibody [129]. In this report, 1 patient had partial remission (PR) and 2 had long stable disease (SD) more than 6 months. More recently, we also reported

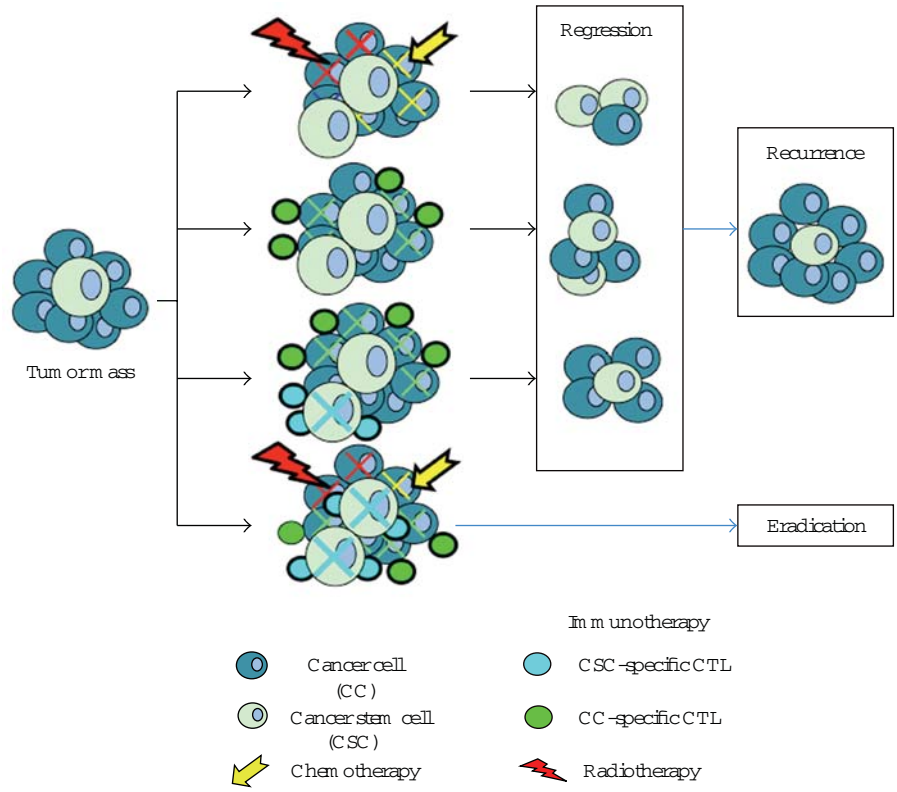

Figure 5: Combination therapies of immunotherapy and standard radio- and chemotherapy. Currently applied standard therapies such as radio- and chemotherapy target bulk cancer cells that are less resistant than cancer stem cells. This leads to initial regression of the tumor mass but eventually regrowth from residual CSCs. Combined therapies of standard therapies and immunotherapeutic approach targeting CSCs would cut off the rejuvenating supply of CSCs and resulted in tumor eradication.

that DC vaccine-based immunotherapies combined with gemcitabine/S-1 were effective in patients with advanced pancreatic cancer refractory to standard chemotherapy [94]. As both WT1 and MUC1 are one of the excellent TAAs for the target of immunotherapy and are frequently expressed in pancreatic cancer cells [36,37,123,130], 38 out of 49 patients had received vaccination with WT1 peptide-pulsed DCs with or without combination of other peptides such as MUC1, CEA, and CA125 in this report. Prior to this combination therapy, 46 out of 49 patients had been treated with chemotherapy, radiotherapy, heavy particle radiotherapy, or hyperthermia but elicited no significant effects. In spite of these handicapped conditions, surprisingly, of 49 patients, 2 patients showed CR, 5 PR, and 10 SD, and median survival time was 360 days. The use of DCs-based vaccines in direct combination with chemotherapy in patients with pancreatic cancer might become a veritable option for the treatment of patients with advanced-stage cancer. Indeed, gemcitabine enhanced WT1 expression in human pancreatic cancer cells and sensitized the pancreatic cancer cells with WT1-specific T cell-mediated antitumor responses [131]. Although the concept is far from being firmly established, these reports may be sufficient to provide a platform for the combination of immunotherapy with chemotherapy. Evaluation is warranted to examine the effect of the approach on disease-free survival and overall survival.

Disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the paper.

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Clinical Study

Comparative Approach to Define Increased Regulatory T Cells in Different Cancer Subtypes by Combined Assessment of CD127 and FOXP3

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In recent years an increase of functional $CD4^+CD25^+$ regulatory T cells (T_{reg} cells) has been established for patients with solid tumors, acute leukemias, and lymphomas. We have reported an expanded pool of $CD4^+CD25^{high}$ T_{reg} cells in patients with chronic lymphatic leukemia (CLL), multiple myeloma (MM) as well as its premalignant precursor monoclonal gammopathy of undetermined significance (MGUS). In healthy individuals, low-level expression of CD127 on T cells in addition to the expression of FOXP3 has been associated with T_{reg} cells. Here, we demonstrate that the expanded $FOXP3^+$ T-cell population in patients with colorectal cancer, CLL, MGUS, MM, follicular lymphoma, and Hodgkin's disease are exclusively $CD127^{low}$ T_{reg} cells and were strongly suppressive. A significant portion of $CD127^{low}FOXP3^+$ T_{reg} cells expressed only low levels of CD25 suggesting that the previously reported expansion of $CD25^+$ T_{reg} cells underestimates the true expansion. The assessment of CCR7 and CD45RA expression on the expanded $CD4^+CD127^{low}FOXP3^+$ T_{reg} cells revealed an increase of both naïve as well as central and effector memory T_{reg} cells in peripheral blood. Our data strongly support superiority of combined CD127 and FOXP3 analysis in comparison to CD25 and FOXP3 assessment for further quantification of T_{reg} cells in malignant diseases.

1. Introduction

$CD4^+CD25^+$ regulatory T cells (T_{reg} cells) are expanded in murine tumor models, and their deletion can lead to complete tumor regression [1]. In humans, T_{reg} cells are mostly enriched in the $CD4^+CD25^{high}$ T-cell population [2]. We and others have reported increased frequencies of $CD4^+CD25^{high}FOXP3^+$ T_{reg} cells in cancer patients [1, 3]. However, the expansion of T_{reg} cells based on the assessment of CD25 is likely to underestimate the true expansion since $FOXP3^+$ T cells are also present in the $CD25^{-/low}$ fraction [4, 5]. Furthermore, molecular and functional characterization of this population is hampered by the inability to separate $CD25^+$ T_{reg} cells from activated effector T cells. Two recent studies, however, have shown that reciprocal expression of the IL7 receptor (CD127) on $FOXP3^+$ T_{reg} cells is most likely a more specific way to quantify $FOXP3^+$ T_{reg} cells [5, 6]. This has been adopted lately for the quantification of T_{reg} cells in solid tumors [7–10] and hematologic malignancies [11–13], with one of the reports establishing CD127 as an even superior marker for the identification of T_{reg} cells in cancer patients [9]. However, no systematic analysis has been undertaken to establish CD127 as a superior marker for T_{reg}-cell enumeration in cancer patients, and only one initial report of malignant melanoma patients has addressed reciprocal expression of CD127 and FOXP3 on T_{reg} cells in cancer patients independently of CD25 [9]. It is, therefore, necessary to determine whether CD127 is also a better marker for enumerating $FOXP3^+$ T_{reg} cells in cancer patients in general by comparing T_{reg} cells numbers in a larger number of different tumor subtypes. Besides the integration of $CD25^{low/-}$ FOXP3-expressing T_{reg} cells, analysis of CD127

might, furthermore, clarify contradictory results concerning frequencies as well as prognostic value of T_{reg} cells in cancer patients [14–16].

Similarly, there is still debate whether human $CD4^+CD25^{high}FOXP3^+$ T_{reg} solely belong to the memory T-cell compartment [17]. Valmori et al. were the first to identify a T_{reg}-cell population with a naïve phenotype ($CCR7^+CD45RA^+$), which they termed natural naïve T_{reg} cells [18]. As expected, the frequency of these naïve T_{reg} cells was relatively low in healthy individuals [19]. More recently, Seddiki et al. have described the persistence of a population of naïve $CD45RA^+$ T_{reg} cells in adult life [20], which was further characterized by resistance to CD95L-induced cell death [21]. Recent data further supports that a population of naïve T_{reg} cells exist in healthy individuals that exerts suppressive function [22]. So far, our own observations suggested an increased frequency of naïve $CD4^+CD25^{high}FOXP3^+$ T_{reg} cells in MM and MGUS [23]. However, previous findings were restricted to the $CD4^+CD25^{high}$ subpopulation excluding a significant fraction of T_{reg} cells from analysis. With the emergence of CD127 as a new marker separating T_{reg} cells from conventional T cells, the question whether the expanded T_{reg} cells in cancer patients are mainly antigen-experienced memory cells or also naïve T_{reg} cells needs reevaluation.

Here, we present clear evidence that $FOXP3^+$ T cells derived from patients with CLL, MGUS, MM, follicular lymphoma (FL), Hodgkin's disease (HD), and colorectal cancer (CRC) are lacking CD127. This newly defined fully functional $CD4^+CD127^{low}FOXP3^+$ T_{reg}-cell population is expanded in all tumor entities as well as the premalignant MGUS supporting the hypothesis of increased T_{reg} cells as a rather early event during tumor development. Moreover, we demonstrate a significant increase of naïve $CD4^+CD127^{low}FOXP3^+$ T_{reg} cells in peripheral blood of cancer patients while we could not detect an increase in lymph node biopsies of lymphoma patients. Finally, these data strongly support the assessment of CD127 expression—instead of CD25—in combination with FOXP3 for a more precise enumeration of T_{reg} cells in malignant diseases while functional characterization still relies on the combination of CD127 and CD25.

2. Material and Methods

2.1. Patients and Clinical Parameters. Following approval by the institutional review board of the University of Cologne, peripheral blood from 10 healthy individuals, 7 MGUS, 10 MM, 10 CLL and 6 patients with CRC (2 time points at least 1 month apart) was obtained after informed consent. For the assessment of T_{reg} cell numbers in lymph node biopsies, lymph nodes from 7 healthy donors, 6 patients with HD, and 7 patients with FL were analyzed following approval by the institutional review board of the University of Cologne. Patients were either untreated or had not received cytoreductive treatment for a period of at least 1 month prior to investigation. Characteristics of the patients studied are summarized in Tables 1 and 2.

2.2. Antibodies and FACS Analysis. Phenotype of T cells was defined by flow cytometry using the following antibodies: CD45RA-PE-Cy5 (HI100), CD127-PE (hIL-7R-M21), CD4-APC-Cy7 (RPA-T4), CD25-PE-Cy7 (M-A251, all from Becton Dickinson), CCR7-FITC (150503, R&D) as well as the corresponding isotype control antibodies. Intracellular staining for FOXP3 was performed with FOXP3-APC (PCH101, eBioscience) according to the manufacturer's recommendations [23]. Samples were acquired on a FACS LSR II and analyzed with FlowJo software (TreeStar Inc). Frequencies of $CD4^+CD127^{low}FOXP3^+$ T cells are shown as percent values of $CD4^+$ T cells.

2.3. Isolation of $CD4^+CD127^{low}CD25^{+/low}$ and $CD4^+CD127^+CD25^-$ T Cells and Assessment of Inhibitory Function. To assess the suppressive activity of $CD4^+CD127^{low}CD25^{+/low}$ T cells, a modified MLR was performed as previously described [23]. Briefly, $CD4^+CD127^{low}CD25^{+/low}$ and $CD4^+CD127^+CD25^-$ T cells were stained with CD4, CD25, and CD127 mAb and sorted on a FACSDiVa or FACS Aria III (both BD Biosciences) and incubated for 20 hours with 10 U/mL IL-2 (Proleukin) and 0.5 μg/mL anti-CD3 (OKT3) in X-VIVO 15 (BioWhittakker) [24]. Subsequently, $CD4^+CD127^+CD25^-$ T cells were stained with 5,6-Carboxyfluorescin-Diacetat-Succinimidyl-Ester (CFSE, Sigma-Aldrich) and stimulated in X-VIVO 15 supplemented with 10% fetal calf serum, 100 U/mL penicillin/streptomycin and 2 mM glutamine (Invitrogen) with magnetic beads (Dynal Biotech,) coated with 5% anti-CD3, 14% anti-CD28 (9.3), and 81% anti-MHC class I (W6/32) at a ratio of 3 : 1 (cells : beads). To assess inhibitory capacity of T_{reg} cells from cancer patients, autologous $CD4^+CD127^{low}CD25^{+/low}$ T_{reg} cells were added at a 1 : 1 ratio to the culture, and the proliferation of $CD4^+CD127^+CD25^-$ T cells was determined by assessing CFSE dilution after four days of culture as described previously [23].

2.4. RNA Preparation and Quantitative Real-Time PCR. For analysis of CD127 mRNA expression, $CD4^+CD25^-$ and $CD4^+CD25^{high}$ T cells from five healthy donors and five CLL patients were purified as previously described [24]. The described technique is optimized for the isolation of human $CD4^+CD25^{high}$ T cells with high purity [23, 24]. Cells were reanalyzed after sorting and routinely showed >95% purity. Subsequently, the cells were lysed in TRIzol reagent (Invitrogen). 50–100 ng RNA were reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Penzberg, Germany). Rt-PCR was performed with the LightCycler Taqman master kit and Universal ProbeLibrary Assay on a Light Cycler 480 II. Analysis was performed using Light-Cycler 3 and RelQuant software using a calibrator normalized relative quantification based on β-2 microglobulin (B2M) expression. Primers used: CD127 forward, 5′-AAAGTTTTAATGCACGATGTAGCTT-3′; CD127 reverse, 5′-TGTGCTGGATAAATTCACATGC-3′; Probe 72; B2M forward, 5′-TTCTGGCCTGGAGGCTAT-3′; B2M reverse, 5′ TCAGGAAATTTGACTTTCCATTC-3′; Probe 42.

Table 1: Patient characteristics for T_{reg}-cell assessment in peripheral blood.

(a) MGUS (peripheral blood)

ID	Gender	Age (yr)	Stage	Therapy	Paraprotein	T_{reg} (%)
1	M	37	MGUS	no tx	IgG/	3.6
2	F	84	MGUS	no tx	IgA/	7.0
3	F	86	MGUS	no tx	IgG/	7.5
4	F	62	MGUS	no tx	IgA	6.0
5	M	52	MGUS	no tx	n.a.	6.8
6	F	79	MGUS	no tx	n.a.	2.4
7	M	65	MGUS	no tx	IgM/	8.0

(b) Multiple myeloma (peripheral blood)

ID	Gender	Age (yr)	Stage	Therapy	Paraprotein	T_{reg} (%)
8	M	62	MM IA	M	IgG/	16.5
9	F	39	MM IA	VAD,CAD,M,BMT	IgG/	7.4
10	M	59	MM IIA	no tx	IgG/	10.2
11	F	74	MM IA	no tx	IgG/	4.5
12	M	86	MM IA	no tx	IgG/	6.7
13	M	60	MM IIA	VAD,CAD,M,BMT	IgG/	16.9
14	F	52	MM IIA	VID,C	IgG/	4.4
15	M	59	MM IIA	TAD,CAD,M,BMT	IgG/	8.6
16	F	67	MM IIA	VAD,CAD,M,BMT	IgG/	22.2
17	M	53	MM IIA	no tx	IgG/	8.0

(c) Chronic lymphocytic leukemia (peripheral blood)

ID	Gender	Age (yr)	Stage	Therapy	T_{reg} (%)
18	m	72	CLL A	no tx	6.1
19	m	62	CLL A	no tx	5.7
20	f	73	CLL A	no tx	5.6
21	m	60	CLL B	no tx	9.8
22	f	55	CLL B	no tx	10.6
23	m	73	CLL B	no tx	16.4
24	m	64	CLL B	no tx	7.4
25	m	39	CLL C	no tx	15.3
26	m	60	CLL C	no tx	6.2
27	m	54	CLL C	no tx	6.4

(d) Colorectal cancer (peripheral blood)

ID	Gender	Age (yr)	Stage	Primary tumor	Sites of metastases	T_{reg} (%)
28	f	43	D	Rectum	Liver, bone, pararectal, para-aortal lymph nodes	7.5/10.4
29	f	32	D	Colon	Liver, spleen, ovaries, pelvis, peritoneum	5.7/9.3
30	m	57	D	Rectum	Lung	5.7/9.9
31	m	66	D	Colon	Liver	5.0/6.2
32	m	75	D	Colon	Liver	9.0/4.8
33	f	44	D	Colon	Liver	9.9/3.3

(e) Healthy donors (peripheral blood)

ID	Gender	Age (yr)	T_{reg} (%)
34	m	66	5.4
35	m	67	6.6
36	m	55	3.9
37	m	50	6.5
38	m	47	4.5
39	m	46	3.5

(e) Continued.

ID	Gender	Age (yr)	T_{reg} (%)
40	m	46	4.8
41	m	62	4.1
42	m	45	2.6
43	f	44	4.0

Patient characteristics including gender, age at analysis, Durie and Salmon, Binet or Dukes stage, first diagnosis, primary tumor, sites of metastases, therapy, paraprotein, and frequency of T_{reg} cells. (f: female, m: male; therapy: A: Doxorubicin, BMT: autologous bone-marrow transplantation, C: Cyclophosphamide, I: Idarubicin, M: Melphalan, V: Vincristine, D: Prednisone, T: Thalidomide, no tx: no therapy, n.a.: not accessible.)

Table 2: Patient characteristics for T_{reg}-cell assessment in lymph node biopsies.

(a) Follicular lymphoma (lymph node)

ID	Gender	Age (yr)	Stage	Therapy	T_{reg} (%)
44	m	59	FL I	no tx	23.9
45	m	46	FL I	no tx	13.4
46	f	58	FL I	no tx	26.8
47	m	73	FL II	no tx	19.6
48	f	66	FL II	no tx	20.0
49	m	59	FL II	no tx	13.2
50	m	57	FL II	no tx	22.5
51	m	65	FL II	no tx	37.5

(b) Hodgkin's disease (lymph node)

ID	Gender	Age (yr)	Entity	Therapy	T_{reg} (%)
52	f	53	HD (ns)	no tx	37.8
53	m	44	HD (ns)	no tx	16.8
54	m	51	HD (ns)	no tx	43.9
55	m	19	HD (ns)	no tx	18.5
56	m	34	HD (ns)	no tx	16.9
57	f	25	HD (mc)	no tx	12.4

(c) Healthy donors (reactive lymph nodes)

ID	Gender	Age (yr)	T_{reg} (%)
58	m	35	4.9
59	f	18	11.7
60	f	17	4.9
61	m	22	9.7
62	f	45	16.7
63	m	39	8.9
64	m	24	14.0

Patient characteristics including gender, age at analysis, first diagnosis, therapy, and frequency of T_{reg} cells. (f: female, m: male; no tx: no therapy; mc: mixed cellularity; ns: nodular sclerosing).

2.5. Statistical Analysis. Comparison between paired or unpaired groups was performed using the appropriate Student's t-test. A P-value < 0.05 was defined as statistically significant. Due to the explorative nature of this study, no multiplicity adjustment procedures were performed. All statistical analyses were performed using the SPSS statistical software package (SPSS 19.0, SPSS Inc.).

3. Results

3.1. Downregulation of CD127 mRNA Expression in $CD4^+CD25^{high}$ T_{reg} Cells from CLL Patients. As CD25 is not solely expressed on T_{reg} cells but also on activated conventional $CD4^+$ T cells, and the downregulation of CD127 expression in $CD4^+CD25^{high}$ $FOXP3^+$ T_{reg} cells from healthy donors has been reported [5, 6], we first assessed if CD127 downregulation is also apparent in $CD4^+CD25^{high}$ T_{reg} cells from cancer patients. We detected a significant downregulation of CD127 mRNA expression in $CD4^+CD25^{high}$ T cells from healthy donors ($n = 5$) as well as CLL patients ($n = 5$, $P < 0.05$, Figure 1(a)) by quantitative PCR indicating that CD127 expression might also be used to specifically identify $CD4^+FOXP3^+$ T_{reg} cells in cancer patients.

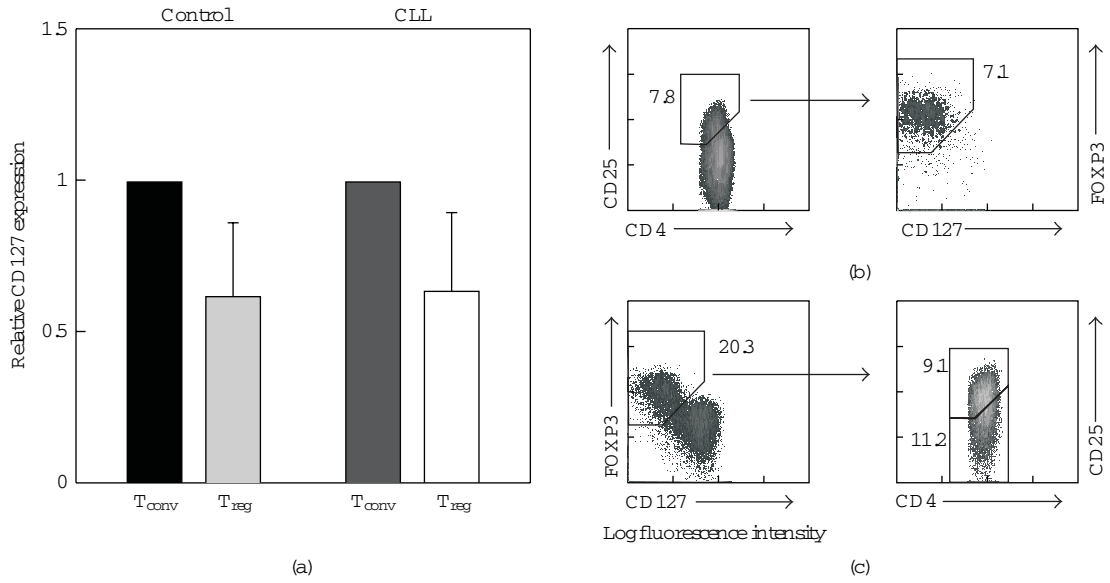

Figure 1: CD127 mRNA expression in $CD4^+CD25^{high}$ T_{reg} cells and integration of CD127 in the analysis of human T_{reg} cells. (a) Expression of CD127 mRNA in $CD4^+CD25^{high}$ T_{reg} cells and conventional $CD4^+CD25^-$ T cells in healthy donors ($n = 5$, control) and CLL patients ($n = 5$, CLL) as determined by qPCR (, $P < 0.05$, Student's t-test). (b) Gating strategies for analysis of expression of CD127 in $CD4^+CD25^{high}FOXP3^+$ T_{reg} cells or (c) CD25 expression in $CD4^+CD127^{low}FOXP3^+$ T_{reg} cells.

3.2. Coassessment of CD127 and FOXP3 for the Enumeration of Human T_{reg} Cells. Next the expression of CD127 in relation to FOXP3 and CD25 was evaluated by flow cytometry on $CD4^+$ T cells. Gating on CD4 and CD25 with subsequent analysis of the $CD4^+CD25^{high}$ T_{reg}-cell population for expression of FOXP3 and CD127 confirmed the downregulation of CD127 in $CD4^+CD25^{high}FOXP3^+$ T_{reg} cells on protein level in healthy individuals (Figure 1(b)). However, assessing coexpression of CD127 and FOXP3 by $CD4^+$ T cells without gating beforehand on the $CD4^+CD25^{high}$ T-cell population clearly revealed a significantly higher percentage of cells expressing FOXP3 but lacking CD127 (Figure 1(c)). Subsequent analysis of the $CD127^{low}FOXP3^+$ T_{reg}-cell population for expression of CD25 demonstrated that gating on CD127 and FOXP3 identifies not only $CD4^+CD25^{high}$ T_{reg} cells but also T_{reg} cells expressing only low levels of CD25 (Figure 1(c)). The identification of this subpopulation of T_{reg} cells is of specific interest as up to now only T_{reg} cells expressing high amounts of CD25 were accessible to functional analysis.

3.3. Increase of $CD4^+CD127^{low}FOXP3^+$ T_{reg} Cells in Cancer Patients. Inclusion of the $CD25^{low}$ T_{reg}-cell subpopulation in the enumeration of T_{reg} cells by defining human T_{reg} cells as $CD4^+CD127^{low}FOXP3^+$ demands the reassessment of T_{reg}-cell frequencies in cancer patients as the actual frequencies were probably underestimated until now. Comparison of healthy individuals with cancer patients revealed elevated levels of $CD4^+CD127^{low}FOXP3^+$ T_{reg} cells in cancer and MGUS patients, as exemplified for individual patients in Figures 2(a) and 2(b). In total, frequencies of T_{reg} cells derived from peripheral blood of 12 patients with CRC, 10 CLL patients, 7 MGUS, and 10 MM patients as well as 10 healthy individuals were evaluated. In addition, lymph node biopsies from 7 patients with follicular lymphoma, 6 patients with Hodgkin's disease, and 7 reactive lymph nodes from healthy individuals were assessed for expanded T_{reg}-cell numbers. Gating on CD4 and CD25 with subsequent gating on FOXP3 confirmed the already described increase of T_{reg} cells in patients with CRC, CLL, MGUS, MM, FL, and HD (Figures 3(a) and 3(b) and Tables 3 and 4). More important, when gating on FOXP3 and CD127 without using CD25 as primary inclusion criteria, frequencies of $CD4^+CD127^{low}FOXP3^+$ T_{reg} cells in controls ($4.1\% \pm 0.7\%$) were similar to previously published results (Figure 3(c) and Table 3) [2, 5, 6, 24]. In contrast, individuals with CRC ($7.2\% \pm 2.4\%$, $P < 0.005$), CLL ($8.9\% \pm 4.0\%$, $P < 0.005$), as well as MM ($11.7\% \pm 5.4\%$, $P < 0.005$) showed significantly increased frequencies of $CD127^{low}FOXP3^+$ T_{reg} cells compared to healthy individuals (Figure 3(c) and Table 3). Even in MGUS patients, a significantly higher frequency of T_{reg} cells ($6.0\% \pm 1.8\%$, $P < 0.05$) was observed (Figure 3(c) and Table 3), which is indicative of T_{reg}-cell expansion as an early event in tumorigenesis. Similarly, we observed significantly increased frequencies of $CD127^{low}FOXP3^+$ T_{reg} cells in patients with FL ($21.8\% \pm 8.0\%$, $P < 0.01$) and HD ($24.4\% \pm 13.1\%$, $P < 0.05$) in comparison to reactive lymph node specimens from healthy individuals ($10.1\% \pm 4.4$, Figure 3(d) and Table 4). Moreover, the percentage of $FOXP3^+$ cells within the $CD4^+CD127^{low}$ T-cell population was always higher than within the $CD4^+CD25^{high}$ population, suggesting that previous data only assessing a $CD4^+CD25^{high}$ phenotype have underestimated the absolute increase of $FOXP3^+$ T_{reg} cells in cancer patients (Tables 3 and 4).

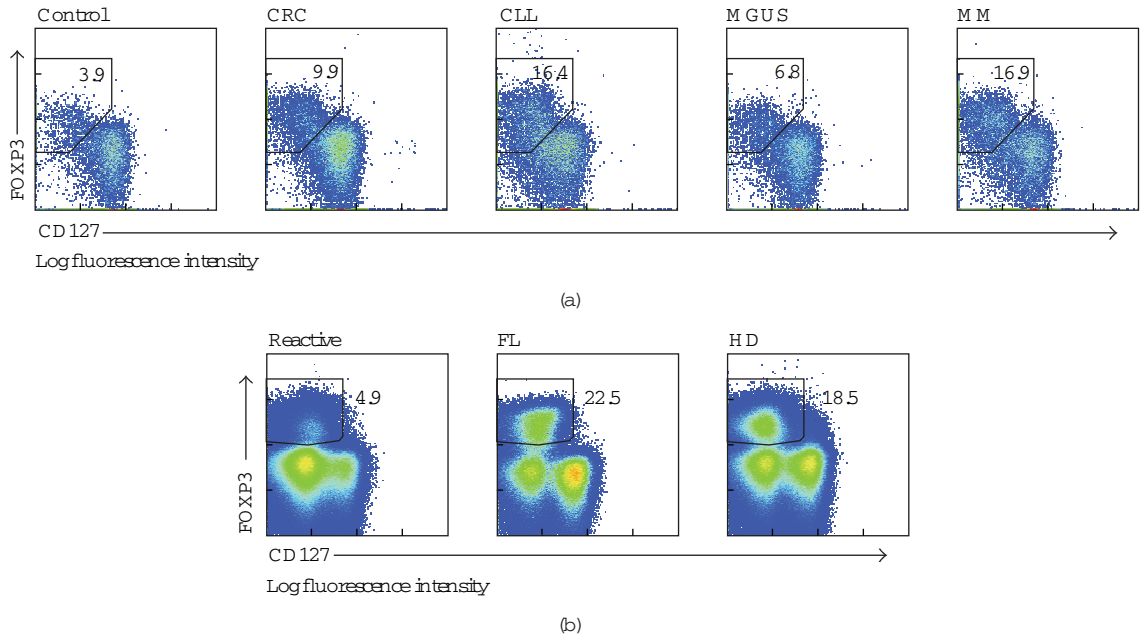

Figure 2: Frequency of $CD4^+CD127^{low}FOXP3^+$ T_{reg} cells. Flow cytometric analysis of CD127 and FOXP3 expression in $CD4^+$ T cells from (a) peripheral blood of a representative healthy individual (control) and representative patients with colorectal cancer (CRC), CLL, MGUS, and multiple myeloma (MM) and (b) lymph node biopsies from a healthy individual (reactive) and patients with follicular lymphoma (FL) and Hodgkin's diseases (HD).

Table 3: Assessment of T_{reg}-cell frequencies in peripheral blood.

	Control	Colon		CLL		MGUS		MM	
	Mean (SD)	Mean (SD)	P	Mean (SD)	P	Mean (SD)	P	Mean (SD)	P
$CD4^+CD127^{low}$	4.6 (1.3)	7.6 (1.5)	<0.001	8.3 (2.5)	<0.005	5.4 (1.3)	n.s.	11.2 (5.9)	<0.01
$CD4^+CD127^{low}FOXP3^+$	4.1 (0.7)	7.2 (2.4)	<0.005	8.9 (4.0)	<0.005	6.0 (1.8)	<0.05	11.7 (5.4)	<0.005
$CD4^+CD127^{low}FOXP3^+CD25^{high}$	2.5 (0.6)	4.3 (1.6)	<0.005	4.7 (2.7)	<0.05	3.9 (1.3)	<0.05	7.1 (4.9)	<0.05
$CD4^+CD25^{high}$	2.8 (0.9)	7.6 (1.2)	<0.001	6.4 (1.8)	<0.001	4.5 (1.1)	<0.05	9.0 (5.3)	<0.01
$CD4^+CD25^{high}CD127^{low}$	2.9 (0.9)	4.5 (1.2)	<0.005	4.5 (2.0)	<0.05	3.5 (1.3)	n.s.	7.0 (5.1)	<0.05
$CD4^+CD25^{high}FOXP3^+$	2.1 (0.8)	4.2 (1.2)	<0.001	3.6 (1.7)	<0.05	2.5 (0.6)	n.s.	6.3 (4.5)	<0.05
$CD4^+FOXP3^+$	2.8 (0.9)	4.7 (2.1)	<0.05	4.7 (2.4)	<0.05	3.6 (1.1)	n.s.	7.7 (5.1)	<0.05

Definition of subpopulations based on expression of CD25, CD127, and FOXP3 (SD: standard deviation, n.s.: not significant).

3.4. $CD4^+CD127^{low}CD25^{+/low}$ T_{reg} Cells are Fully Functional in Cancer Patients. As intracellular FOXP3 staining is not applicable for functional analysis of T_{reg} cells, classification of $FOXP3^+$ T_{reg} cells based solely on cell surface markers is necessary. The characterization of $FOXP3^+$ T_{reg} cells was best achieved when combining CD127 and CD25 (Figures 4(a) and 4(b)). We, therefore, used this combination of cell surface markers to sort T_{reg} cells for functional analysis. Staining for FOXP3 expression after sorting routinely showed purities of $CD4^+CD127^{low}FOXP3^+CD25^{+/low}$ T_{reg} cells >95 percent (Figure 4(b)). To determine whether the $CD4^+CD127^{low}CD25^{+/low}$ T_{reg} cells from cancer patients are functional, we used an in vitro suppression assay. When activated with CD3/CD28 beads conventional $CD4^+CD127^+CD25^-$ T cells, but not $CD4^+CD127^{low}CD25^{+/low}$ T_{reg} cells, proliferate strongly. In the presence of $CD4^+CD127^{low}CD25^{+/low}$ T_{reg} cells, this proliferation is suppressed (Figure 4(c)). These data clearly demonstrate that $CD4^+CD127^{low}CD25^{+/low}$ T cells are $FOXP3^+$ and that these cells are fully functional in CRC patients.

3.5. Naïve $CD4^+CD127^{low}FOXP3^+$ T_{reg} Cells are Increased in Peripheral Blood of Cancer Patients. In healthy individuals, T_{reg} cells have been shown to exist at all differentiation states, namely, naïve, central, and effector memory T_{reg} cells [18, 20, 25]. To determine which T_{reg}-cell subpopulation is responsible for the increase of $CD4^+CD127^{low}FOXP3^+$ T_{reg} cells in cancer patients, we determined the frequency of naïve, central, and effector memory cells within the T_{reg}-cell compartment from healthy individuals, CRC, CLL, MGUS, and MM patients (Figure 5(a)) and compared these data with those previously described for $CD4^+CD25^{high}$ T_{reg} cells in healthy individuals as well as MGUS and MM patients [18, 23]. In healthy individuals, naïve $CCR7^+CD45RA^+CD4^+CD127^{low}FOXP3^+$ T_{reg} cells were hardly detectable (Figures 5(b) and 5(c)). T_{reg} cells were

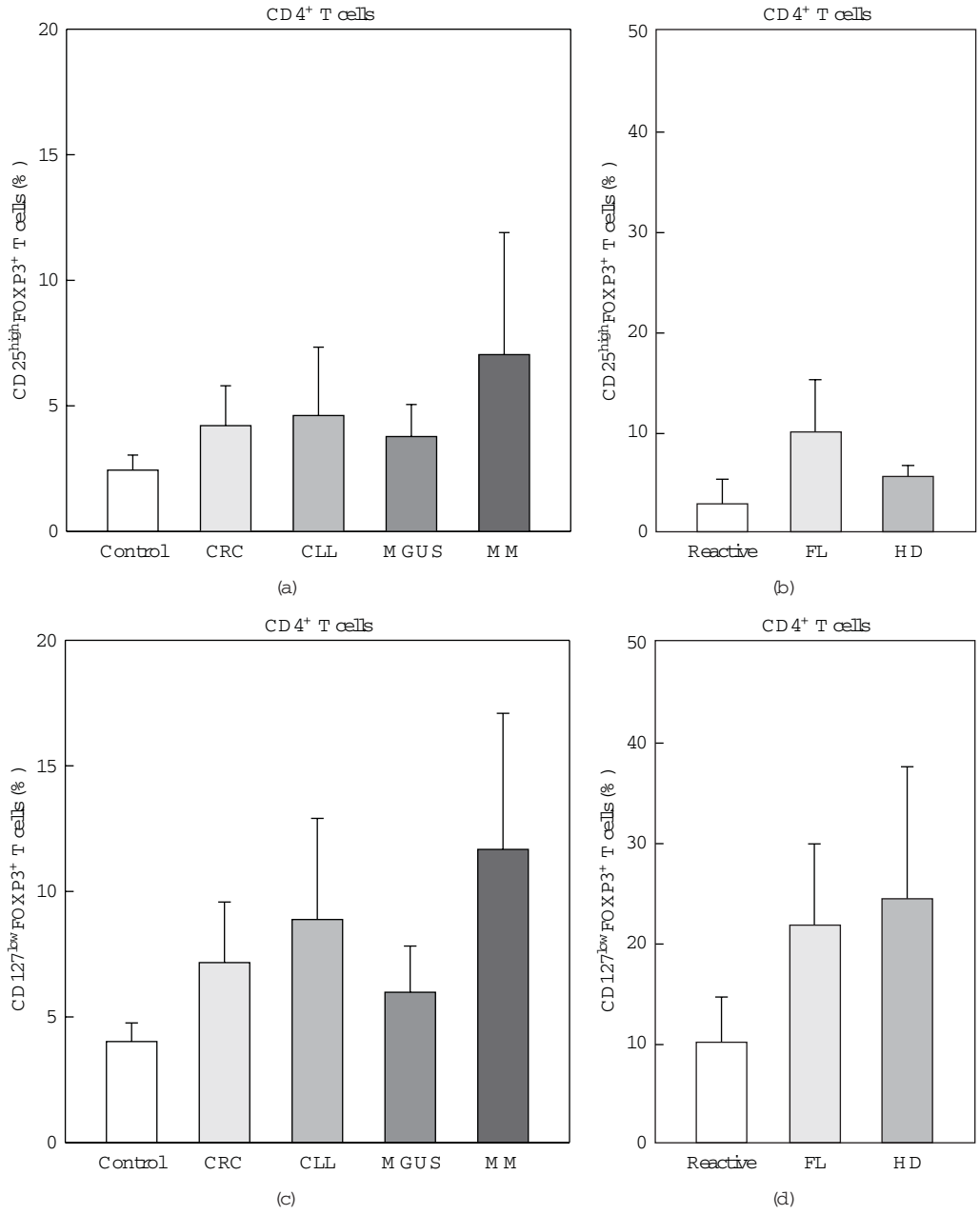

Figure 3: Assessment of T_{reg}-cell frequencies. Frequency of $CD4^+CD25^{high}FOXP3^+$ T_{reg} cells in (a) peripheral blood of 10 healthy donors (control), 12 colorectal cancer (CRC), 10 CLL, 7 MGUS, and 10 multiple myeloma (MM) patients and (b) 7 reactive lymph node biopsies from healthy individuals (reactive), 7 patients with follicular lymphoma (FL), and 6 patients with Hodgkin's disease (HD). (c) and (d) Frequencies of $CD4^+CD127^{low}FOXP3^+$ T_{reg} cells in the respective groups. Error bars represent standard deviation (, $P < 0.05$, Student's t test).

almost exclusively of memory phenotype (Figures 5(b) and 5(c)). In contrast, in peripheral blood of CRC, CLL, and MM patients, a significant expansion of $CD4^+CD127^{low}FOXP3^+$ T_{reg} cells with a naïve phenotype was observed (Figures 5(b) and 5(c)). The expansion of naïve T_{reg} cells was apparent as part of the T_{reg}-cell pool as well as in relation to the total number of $CD4^+$ T cells in cancer patients. This increase in naïve T_{reg} cells was further accompanied by an expansion of T_{reg} cells with a central as well as effector memory phenotype in all patient groups (Figures 5(b) and 5(c)). Interestingly, the observed expansion of naïve $CD4^+CD127^{low}FOXP3^+$ T_{reg} cells was also detectable in MGUS patients (Figures 5(b) and 5(c)) further underlining that frequencies of naïve T_{reg} cells increase rather early during tumor development and progression. When assessing subpopulations of T_{reg} cells in lymph node specimens, we observed a predominance of $CD4^+CD127^{low}FOXP3^+$ T_{reg} cells with a central-memory phenotype, with a significantly

Table 4: Assessment of T_{reg}-cell frequencies in lymph node biopsies.

	Control	FL		HD	
	Mean (SD)	Mean (SD)	P	Mean (SD)	P
$CD4^+CD127^{low}$	54.7 (23.1)	68.6 (15.8)	>0.05	68.0 (12.7)	>0.05
$CD4^+CD127^{low}FOXP3^+$	10.1 (4.4)	21.8 (8.0)	<0.01	24.4 (13.1)	<0.05
$CD4^+CD127^{low}FOXP3^+CD25^{high}$	3.1 (1.9)	11.7 (5.5)	<0.005	6.4 (2.3)	<0.05
$CD4^+CD25^{high}$	5.1 (2.9)	13.8 (7.1)	<0.05	11.2 (3.3)	<0.005
$CD4^+CD25^{high}CD127^{low}$	4.1 (3.0)	16.3 (7.1)	<0.005	11.3 (5.2)	<0.05
$CD4^+CD25^{high}FOXP3^+$	2.9 (2.6)	10.0 (5.4)	<0.01	5.6 (1.2)	<0.05
$CD4^+FOXP3^+$	10.3 (5.5)	19.4 (8.5)	<0.05	23.4 (12.0)	<0.05

Definition of subpopulations based on expression of CD25, CD127, and FOXP3 (SD: standard deviation, n.s.: not significant).

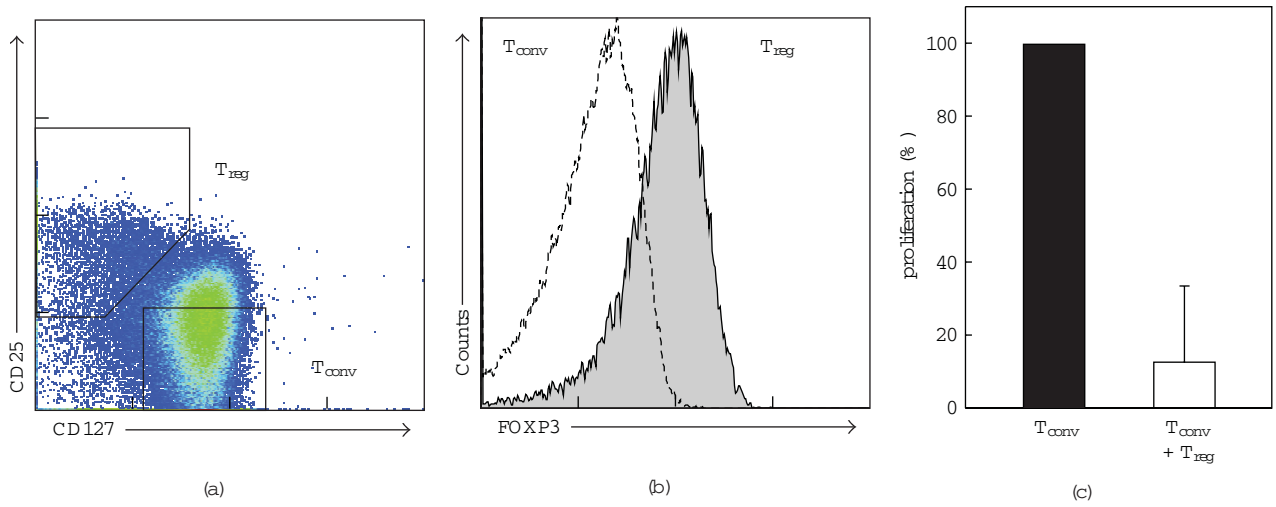

Figure 4: Functional analysis of $CD4^+CD127^{low}CD25^+$ T_{reg} cells in cancer patients. (a) Sorting strategy for isolating $CD4^+CD127^{low}CD25^+$ T_{reg} cells (T_{reg}) as well as conventional $CD4^+CD127^+CD25^-$ T cells (T_{conv}). (b) Expression of FOXP3 in the corresponding T-cell populations. (c) Percentage of proliferation of $CD4^+CD25^-CD127^+$ T_{conv} cells (black bar) alone or cultivated with $CD4^+CD127^{low}CD25^+$ T_{reg} cells derived from CRC patients (n = 4) at a 1 : 1 ratio (white bar) both in the presence of CD3/CD28 mAb coated beads. Error bars represent standard deviation (, $P < 0.05$, Student's t test).

expanded population of central-memory T_{reg} cells apparent in patients with FL and HD (Figure 5(d)). In addition, we could also detect an increase in effector-memory T_{reg} cells (Figure 5(d)) while the pool of naïve T_{reg} cells was basically absent independent if reactive or diseased lymph nodes were analyzed (Figure 5(d)).

4. Discussion

Expansion of $CD4^+CD25^{high}$ T_{reg} cells within the tumor microenvironment and peripheral blood has so far been accepted as a hallmark of cancer [1, 26, 27]. Moreover, augmented T_{reg}-cell frequencies have been linked to tumor stage, prognosis, and survival [1, 26, 27]. We present new evidence that the increase of T_{reg} cells in cancer was even underestimated previously due to suboptimal classification of T_{reg} cells. Integrating analysis of FOXP3 with the cell-surface molecule CD127 clearly demonstrates that significantly higher numbers of $CD127^{low}FOXP3^+$ T_{reg} cells are expanded in cancer patients in general. The assessment of CD127 instead of CD25 is clearly superior in enumerating T_{reg} cells in the diseased state.

Natural T_{reg} cells have been described as $CD4^+CD25^+$ T cells in mice [28], and initial reports in cancer patients relied solely on the assessment of CD4 and CD25 expression for the identification of T_{reg} cells [3, 29]. Only since the identification of the transcription factor FOXP3 lineage-specific marker of T_{reg} cells a more specific characterization of T_{reg} cells is possible [28]. In murine models, FOXP3 expression is strongly associated with the $CD25^+$ T_{reg}-cell population. However, even the inclusion of FOXP3 assessment has been interpreted differentially when assessing frequencies of T_{reg} cells in healthy individuals and cancer patients [23, 30]. The analysis of T_{reg} cells in humans has been further complicated as several studies reported $FOXP3^+$ cells within the $CD4^+CD25^{low}$ or even $CD4^+CD25^-$ population [5], and even the reprogramming of T_{reg} cells into effector T cells has been reported [31]. Therefore, a more specific definition of T_{reg} cells based on unique or additional T_{reg}-cell marker molecules is urgently needed. The introduction of CD127 as a new marker to distinguish T_{reg} cells from

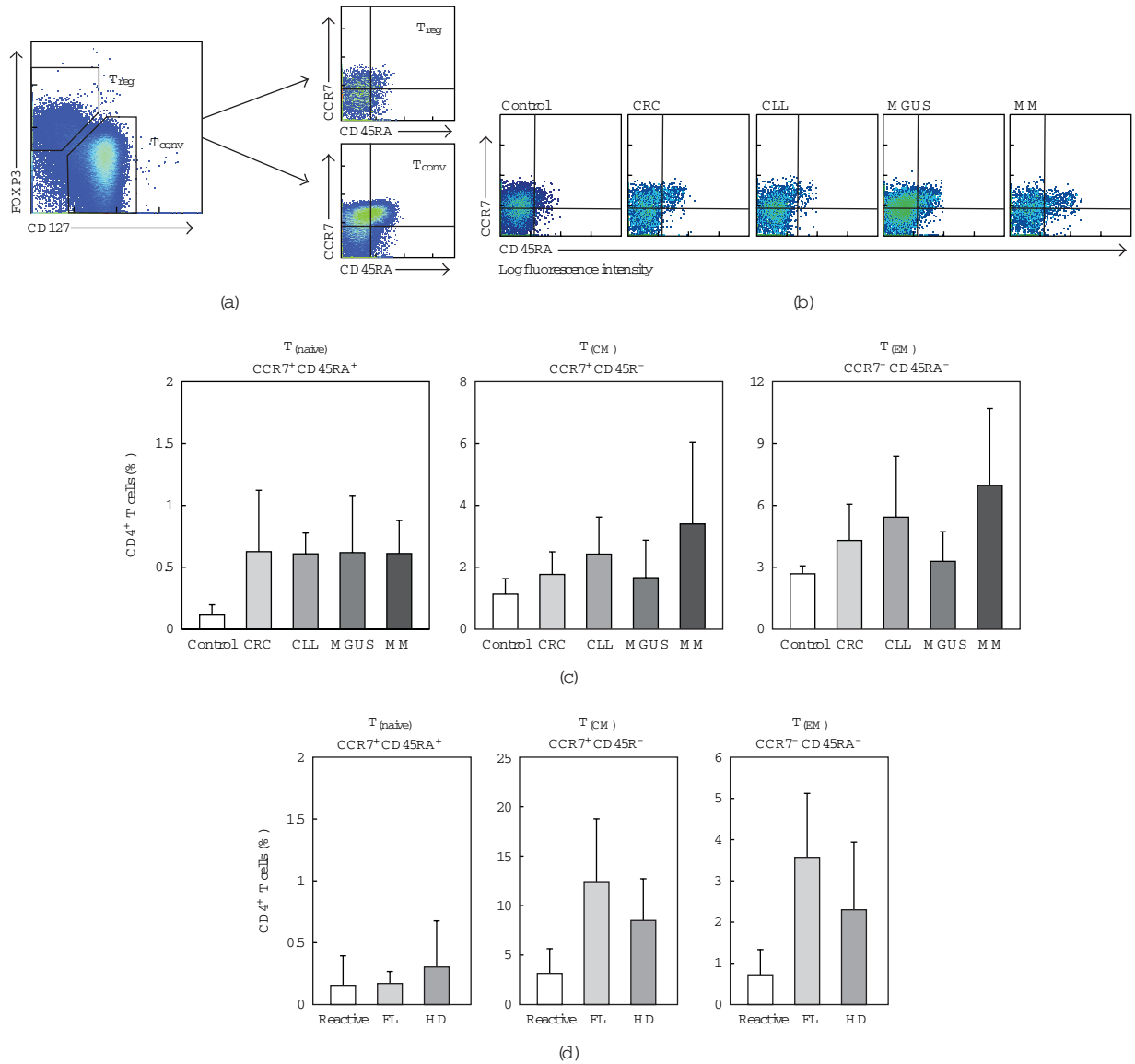

Figure 5: Assessment of naïve CCR7$^+$CD45RA$^+$CD4$^+$CD127^{low}FOXP3$^+$ T_{reg} cells. (a) Frequencies of CCR7$^+$CD45RA$^+$ naïve CD4$^+$CD127^{low}FOXP3$^+$ T_{reg} cells (T_{naive}), CCR7$^+$CD45RA$^-$CD4$^+$CD127^{low}FOXP3$^+$ central memory T_{reg} cells (T_{CM}), and CCR7$^-$CD45RA$^-$CD4$^+$CD127^{low}FOXP3$^+$ effector memory T_{reg} cells (T_{EM}) were assessed in peripheral blood using gating on CD127 and FOXP3 with successive gating on CCR7 and CD45RA. (b) Flow cytometric analysis of naïve, central memory, and effector memory CD4$^+$CD127^{low}FOXP3$^+$ T_{reg} cells in peripheral blood from a representative healthy individual (control) as well as representative patients with colorectal cancer (CRC), CLL, MGUS, and multiple myeloma (MM). Frequencies of regulatory T_{naive}, T_{CM}, and T_{EM} cells were assessed in (c) peripheral blood of CRC (CRC, $n = 12$), CLL (CLL, $n = 10$), MGUS (MGUS, $n = 7$), MM (MM, $n = 10$), and healthy individuals (control, $n = 10$) and (d) 7 reactive lymph node biopsies from healthy individuals (reactive), 7 patients with follicular lymphoma (FL), and 6 patients with Hodgkin's disease (HD). Error bars represent standard deviation (, $P < 0.05$, Student's t-test).

conventional T cells is an important improvement and will help to clarify several previous conflicting results in human T_{reg}-cell biology, particularly in cancer patients.

Several recent studies have adopted the approach to use CD127, CD25, and FOXP3 for the quantification of T_{reg} cells in tumor-bearing individuals and could demonstrate increased numbers of CD4$^+$CD25^{high}CD127^{low} T_{reg} cells in patients with solid tumors [7–10] and hematologic malignancies [11–13]. However, the majority of these reports focused solely on the enumeration of the T_{reg}-cell compartment while at the same time focusing on only one tumor subtype. Only one study assessed T_{reg}-cell numbers in more than one tumor subtype showing similar numbers of T_{reg} cells for all gastrointestinal tumor subtypes analyzed [8]. Furthermore, these studies did not systematically compare possible marker combinations to establish the most suitable approach to identify T_{reg} cells. This was analyzed in more detail in only one of the reports with the combination of CD127 and

FOXP3 being the most appropriate combination to identify T_{reg} cells in patients with malignant melanoma [9].

The integration of CD127 permits to redefine the importance of CD25 expression on human T_{reg} cells. Up to now, high expression of CD25 allowed for an enrichment of $CD4^+$ T cells with regulatory properties [2]. However, it is undisputed that neither all human T_{reg} cells are included by this approach nor that activated T cells expressing CD25 are excluded. Zelenay et al. could demonstrate a population of $CD4^+CD25^-$ FOXP3$^+$ T cells which can upregulate CD25 upon the depletion of all CD25 expressing cells and are able to replace the original T_{reg}-cell population [4]. These data were a first hint that the expression of CD25 on T_{reg} cells is similarly regulated like its expression on conventional T cells [4]. Human T_{reg} cells need IL-2 for their survival and proliferation, and expression of the IL2R- chain is certainly a prerequisite for IL-2 to exert its biological function [32]. However, the expression of CD25 is not homogenous and might also be dependent on the activation status and other exogenous factors [33].

Using CD127 and FOXP3 to define human T_{reg} cells demonstrates varying expression of CD25 in the $CD4^+CD127^{low}$ FOXP3$^+$ T_{reg}-cell population. Additionally, the newly defined T_{reg}-cell population comprises of significantly more T_{reg} cells compared to the traditionally defined $CD4^+CD25^{high}$ T_{reg} cells as demonstrated recently for malignant melanoma [9]. Coassessment of CD127 and FOXP3 to determine T_{reg} cells also resolves the uncertainty to differentiate between activated conventional T cells and T_{reg} cells in patients with active disease. This is of particular importance when only using CD4 and CD25 for the identification of T_{reg} cells in cancer patients, as contamination with effector T cells most frequently occurs when solely these two markers are used for analysis. As functional assessment of the $CD4^+CD127^{low}$ FOXP3$^+$ T_{reg}-cell population is not possible as FOXP3 cannot be used for live studies of human T_{reg} cells, using expression of CD4, CD25, and CD127 is the best possible approximation. T cells isolated by this approach almost exclusively express FOXP3. Moreover, when isolated from cancer patients, this T_{reg}-cell population exerts strong inhibition.

Using a comparative approach analyzing different tumor subtypes from hematologic as well as epithelial origin, we demonstrate that all independent cancer patient groups studied uniformly show an expanded pool of $CD4^+CD127^{low}$ FOXP3$^+$ T_{reg} cells. We therefore postulate that expansion of T_{reg} cells is a general phenomenon in cancer patients. Moreover, since MGUS patients already have increased frequencies of T_{reg} cells, it is very likely that expansion of T_{reg} cells is an early event in the development of human tumors. Elevated T_{reg}-cell levels might be associated with the progression from premalignant lesions that are still under control of the immune system to the uninhibited growth of malignant tumors.

The findings that naïve T_{reg} cells are increased both in the premalignant state as well as in cancer patients might further support this hypothesis. T_{reg} cells were first identified as antigen-experienced memory cells expressing CD45RO [2]. Only recently the existence of naïve T_{reg} cells in human adults has been reported [18, 20, 22, 23, 34], and the naïve T_{reg}-cell population can be expanded in vitro while retaining its suppressive function [35, 36]. However, the physiological function of the naïve T_{reg}-cell population remains unclear. Definition of T_{reg} cells as $CD4^+CD127^{low}$ FOXP3$^+$ has enabled us to verify the increase of naïve T_{reg} cells in MGUS and MM patients [23] and to extend these findings to CLL and CRC.

The identification of an expanded pool of naïve T_{reg} cells in cancer patients opens new avenues to better understand the role of T_{reg} cells in malignant disease. Memory T_{reg} cells apparently cannot undergo self-renewal [37]. Therefore, the replenishment of an increased memory T_{reg}-cell pool by differentiation of naïve T_{reg} cells into memory T_{reg} cells might be an alternative to the recently proposed conversion of conventional memory T cells to T_{reg} cells [37]. In fact, the increased pool of naïve T_{reg} cells with an unaltered frequency of memory T_{reg} cells in premalignant MGUS suggests that expansion of naïve T_{reg} cells is indeed preceding the expansion of memory T_{reg} cells following differentiation during tumor development. Besides the expansion of naïve T_{reg} cells through enhanced self-renewal and differentiation, other mechanisms have been proposed amongst them the interaction of CCR4 on T_{reg} cells with CCL22 released in the tumor microenvironment [38] as well as the conversion of conventional $CD4^+CD25^-$ T cells to T_{reg} cells trough TGF- [39] or prostaglandin E_2 [40]. How these factors influence the expansion of naïve T_{reg} cells needs further clarification and might in the end result in better strategies to target expanded T_{reg} cells in tumor patients.

In conclusion this study demonstrates that $CD4^+CD127^{low}$ FOXP3$^+$ T_{reg} cells are increased in cancer patients. Definition of T_{reg} cells by combining CD127 and FOXP3 has the advantage of including not only T_{reg} cells expressing high levels of CD25 but also T_{reg} cells with low CD25 expression and excluding at the same time activated conventional T cells. Furthermore, the naïve T_{reg}-cell population is expanded in all tumor bearing individuals.

Abbreviations

CLL: Chronic lymphatic leukemia;
MM: Multiple myeloma
MGUS: Monoclonal gammopathy of undetermined significance
CRC: Colorectal cancer
FL: Follicular lymphoma
HD: Hodgkin's disease
FOXP3: Forkhead box protein 3
T_{reg} cells: Regulatory T cells.

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Research Article

Humoral Immune Response to Keyhole Limpet Haemocyanin, the Protein Carrier in Cancer Vaccines

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Keyhole limpet haemocyanin (KLH) appears to be a promising protein carrier for tumor antigens in numerous cancer vaccine candidates. The humoral immune response to KLH was characterized at the single-cell level with ELISPOT combined with separations of cell populations according to their expression of homing receptors (HRs). The analysis of HR expressions is expected to reveal the targeting of the immune response in the body. Eight orally primed and four nonprimed volunteers received KLH-vaccine subcutaneously. Circulating KLH-specific plasmablasts were found in all volunteers, 60 KLH-specific plasmablasts/10^6 PBMC in the nonprimed and 136/10^6 in the primed group. The proportion of L-selectin$^+$ plasmablasts proved high and integrin $_4{}_7{}^+$ low. KLH serving as protein carrier in several vaccines, the homing profile of KLH-specific response may be applicable to the cancer antigen parts in the same vaccines. The present data reflect a systemic homing profile, which appears advantageous for the targeting of immune response to cancer vaccines.

1. Introduction

Many important antigens, whether of cancerous or microbial origin, are poorly immunogenic when injected into humans in a soluble form. However, it is possible to significantly improve their immunogenicity by conjugation to a highly immunogenic protein carrier such as tetanus toxoid (TT) or keyhole limpet haemocyanin (KLH) [1, 2]. This conjugation ensures that the robust T-cell help elicited by the carrier protein is concentrated in the vicinity of T- and B-cells specific to the weak antigen to which the protein carrier is linked. This in turn facilitates the T-B cell cooperation and results in a more vigorous immune response to the weak antigen [2]. This approach has proved highly successful in vaccines containing bacterial polysaccharides conjugated to a protein carrier [1]: pneumococcal and Haemophilus influenzae type b—TT conjugate vaccines, for example, are used widely and have been introduced into national vaccination programmes in several countries. Numerous cancer antigens have been linked with KLH, resulting in promising anticancer vaccine candidates [2–7].

KLH is a naturally occurring immunoadjuvant functioning as a respiratory protein of giant keyhole limpets living in shallow coastal waters in a sea [8, 9]. It is a mixture of two immunologically distinct isoforms, both of which are didecamers assembled from 400 kDa polypeptides; an atomistic model of the quaternary structure of isoform KLH1 is available [8]. This antigen is ordinarily not encountered by the human immune system. KLH has been used in studies addressing the phenomenon of oral tolerance; prolonged oral priming with soluble KLH has been shown to result in a suppression of T-cell-mediated immunity and delayed hypersensitivity reaction after a subsequent subcutaneous immunization [10, 11]. In contrast to this, the humoral

immune response at both mucosal and systemic sites has proved to be enhanced [10]. These studies were carried out to provide tools for eliciting oral tolerance useful in numerous autoimmune diseases.

In addition to the tolerance studies, KLH has been explored in cancer research. Originally, it was found to be cross-reactive with cancer antigens in urinary bladder carcinoma, which led to research into the use of KLH as bladder carcinoma vaccine [2, 9]. More recently, however, KLH has proved successful as a protein carrier to numerous poorly immunogenic cancer antigens such as in vaccines against follicular lymphoma [3], non-Hodgkin lymphoma [6], glioblastoma multiforme [4], melanoma [2], prostate, and ovarian cancer [2]. The conjugate cancer vaccines appear as the most extensively studied application of KLH at the moment.

Immune response to vaccination is not distributed evenly in the body, but, instead, activated lymphocyte populations are guided to traffic only to certain tissues [12, 13]. Tissue-specific migration is based on a multistep process of homing; while the blood carries the activated lymphocytes everywhere in the body, the cells can lodge into tissues only at sites which they recognize by means of their specific surface molecules [12, 13]. The most important molecules determining tissue localization are chemokine (CCR) and homing receptors (HRs) [12, 13], which recognize their ligands in the tissue, chemokines, and endothelial addressins, respectively. Several tissue-specific HRs have been identified; the intestinal HR, $_{4\ 7}$ integrin, guides the cells to the intestinal mucosa [14], L-selectin (CD62L) to peripheral lymph nodes [15, 16] and cutaneous lymphocyte antigen (CLA) to cutaneous sites [17, 18]. We have shown earlier that KLH-specific T-cells after oral feeding express significantly more $_{4\ 7}$ integrin than KLH-specific T-cells after parenteral injection. Thus, the homing profile of KLH-specific T cells depends on the site of antigen encounter [19]. With other antigens, the homing profile of B cells has been found to depend on the site of antigen encounter [20–23]. The homing of B cells after immunization with KLH has not been addressed in any studies before.

The present study characterized at a single-cell level the immune response to KLH and the homing profiles of these cells. KLH being a common protein carrier to numerous cancer antigens in various vaccines [2–7], this homing profile is of special interest, for it can be interpreted as the homing profile associated with all the different injectable cancer vaccines linked to KLH, and thus give an insight into the localization of the response to these vaccines. Optimal targeting of the ensuing immune response should always be one of the goals of vaccine development. As to cancer vaccines in general, it appears most beneficial to target the immune responses to the site where the primary tumor developed.

2. Materials and Methods

2.1. Volunteers. Fourteen healthy volunteers (8 women, 6 men, aged 22–40 years) participated in the study. None of the volunteers had been previously exposed to KLH; all of them had received TT vaccine according to the usual vaccination protocol as a child and a booster dose within the last ten years. The study was approved by the Human Use Committee of the University of Alabama at Birmingham. Informed consent was obtained from each volunteer before participation.

2.2. Experimental Design. Nine fasting volunteers ingested each 100 mg of KLH on days 1 to 5 and days 15 to 19 and were given 100 μg KLH subcutaneously on days 26 and 36 (primed group) (Figure 1(a)). Another 5 volunteers received only the parenteral KLH on days 26 and 36 (nonprimed group). Blood samples were drawn on days 0, 9, and 44. Mononuclear cells were isolated from the blood samples and subjected to immunomagnetic cell selection to sort the cells into receptor-positive and -negative cell populations with respect to their expression of $_{4\ 7}$ integrin and L-selectin (Figure 1(b)). These populations were assayed for KLH-specific ASC with enzyme-linked immunospot (ELISPOT) assay. Due to the limited numbers of cells obtained, all assays could not be carried out on all volunteers.

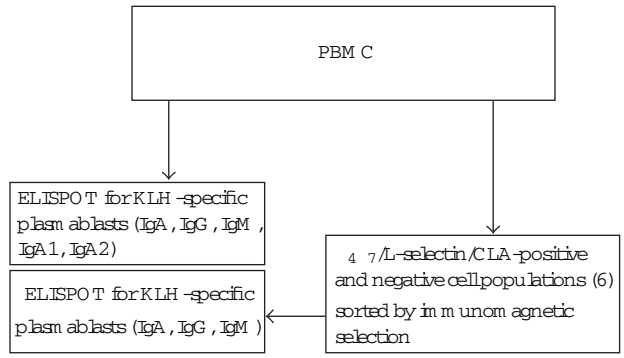
2.3. KLH. KLH as a freeze-dried powder was purchased from Calbiochem Corp. (La Jolla, CA, USA). For oral use, 100 mg of this preparation was packed into gelatin capsules. KLH for parenteral use was purchased from Pacific Biomarine (Venice, CA, USA). It was purified from an ammonium sulphate preparation of the protein. This preparation was dissolved in pyrogen-free saline, passed twice through a polymyxin-agarose column, and assayed for endotoxin content with Limulus assay as described in detail earlier [10].

2.4. Isolation of Mononuclear Cells. Mononuclear cells were isolated with Ficoll-Paque density-gradient centrifugation from heparinized venous blood. The cells were washed twice with PBS and suspended in culture medium (RPMI-1640 supplemented with 10% heat inactivated fetal calf serum, 100 U penicillin/mL and 100 μg streptomycin/mL) [19].

2.5. Separation of the Receptor-Negative and -Positive Cell Populations. The separation of the cells into receptor-negative and -positive populations has been described in detail earlier [20, 22, 24]. Briefly, cells were incubated with one of the first-stage monoclonal antibodies: anti-$_{4\ 7}$ (ACT-1; Millennium Pharmaceuticals, Cambridge, MA, USA) or anti-L-selectin (anti-CD62L; Becton-Dickinson, San Jose, CA, USA). After washing the cells were incubated with Dynal M-450 magnetic beads coated with sheep antimouse IgG (Dynal, Oslo). Receptor-positive and -negative cells were separated with magnetic cell sorting. Both the receptor-positive and -negative cell populations were immediately analyzed with the ELISPOT assay for numbers of all immunoglobulin-secreting cells (ISC) and KLH-specific ASC. The efficiency of the cell separations was checked with flow cytometry in pilot experiments as described earlier [20]; >90% of the L-selectin^{+} cells including all brightly staining cells and >99%

Group	Day 0	Day 1–5	Day 15–19	Day 26	Day 36	Day 44
Primed (n = 9)		KLH p.o.	KLH p.o.	KLH s.c.	KLH s.c.	
Non-primed (n = 5)		—	—	KLH s.c.	KLH s.c.	

(a)



(b)

Figure 1: Experimental design of the study. (a) Protocol for immunization with KLH and for collection of blood samples. The asterisks indicate days of collecting blood samples. (b) Assaying KLH-specific circulating plasmablasts from the blood samples. The cells were assayed both from the total population of PBMC and from receptor-positive and -negative populations (a total of six separated populations for each volunteer) resulting from immunomagnetic sorting with respect to different HR.

of the integrin $_4 _7^+$ cells were removed from the unsorted cells by the negative selection.

2.6. Assay of ISC and ASC (ELISPOT). While ISC represents all plasmablasts in the circulation, antigen-specific plasmablasts represent smaller subpopulations within the whole population of ISC. KLH-specific plasmablasts were identified as ASC specific for KLH. In the ELISPOT assay, plasmablasts are allowed to secrete antibodies in the immediate vicinity of the antigen (capturing Ig in ISC assays and antigen in ASC assays) on a microwell plate, and the antibodies secreted are then detected immunoenzymatically. The substrate is added in hot agarose to immobilize the decaying colour into a spot. Each spot is regarded to correspond to a single-cell secreting antibodies specific to or captured by the coating antigen.

The assays of IgA-, IgG- and IgM-ISC/ASC [25] and IgA1- and IgA2-ISC [26, 27] have been described in detail previously. Briefly, microtiter plates were coated with antisera to human IgA or IgM (Dako, Glostrup, Denmark) or IgG (Sigma, St. Louis, MO, USA) for the ISC-assay, or with KLH (5 μg/mL PBS) for the ASC assay and blocked with 1% bovine serum albumin. The cells were incubated in the wells for 2–3 h, and antibodies secreted during this time were detected with alkaline phosphatase-conjugated antihuman IgA (Sigma-Aldrich), IgG (Sigma-Aldrich) and IgM (Southern Biotech, Birmingham, AL, USA) antisera or, for the IgA-subclass assays, with monoclonal antibodies to IgA1 and IgA2 (Nordic Immunological Laboratories, Tilburg, the Netherlands) followed by alkaline phosphatase-conjugated antimouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Substrate was added to all plates in hot agarose as described in detail earlier [25].

2.7. Statistics. Percentages of cells expressing the different receptors were determined as arithmetic means of the percentages of ISC expressing the given cell surface marker. The proportions of the receptor-positive ISC were calculated as follows: % of receptor-positive cells among ISC = (100 × the number of ASC or ISC in receptor-positive population) ÷ (the sum of the number of ASC or ISC in receptor-positive and receptor-negative populations). In order to get reliable statistics for the proportions of cells expressing a given marker, we set an inclusion limit of 20 ASC that needed to be identified among the cells studied. Statistical analyses were performed with Student's t-test, and the results were considered significant only when $P < .05$.

3. Results

3.1. Numbers of Total Ig-, IgA1-, and IgA2-Immunoglobulin-Secreting Cells (ISC). The total populations of ISC represent the sum of numerous antigen-specific populations of ASC. On the average, 0.2% of peripheral blood mononuclear cells (PBMC) were ISC (plasmablasts). The geometric mean of IgA-ISC was 864, IgG-ISC 800 and IgM-ISC 89 cells/10^6

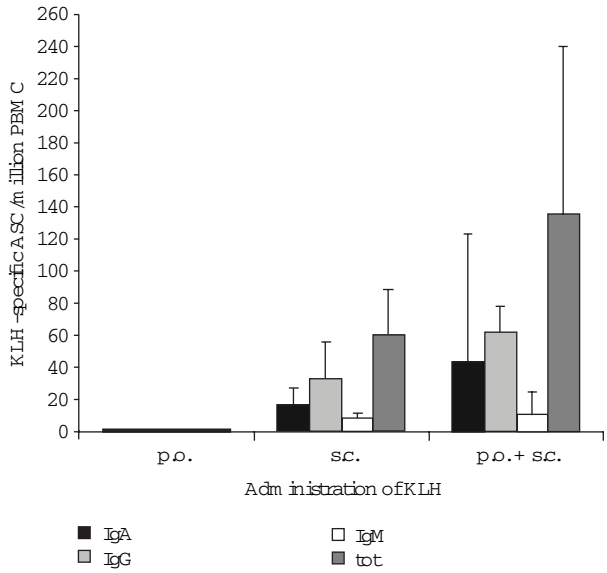


Figure 2: The numbers of KLH-specific plasmablasts identified with ELISPOT as KLH-specific antibody-secreting cells (ASC) in the circulation of vaccinees after oral KLH feeding (n = 9) or after two subcutaneous KLH injections of nonprimed (n = 5) or orally primed (n = 9) volunteers. The data are given as geometric means of ASC/10^6 PBMC ± SEM.

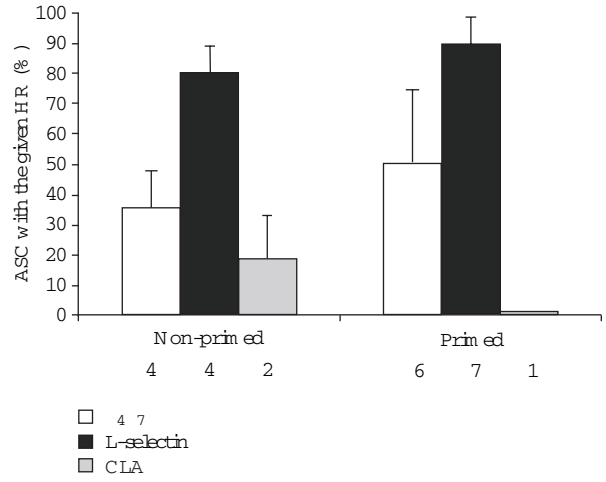


Figure 3: The expression of the intestinal homing receptor, $\alpha_4\beta_7$ integrin, the peripheral lymph node HR, L-selectin, and the skin HR, CLA, on KLH-specific plasmablasts after two subcutaneous KLH injections given to orally primed or nonprimed volunteers. The data were calculated by counting the proportion of HR positive cells among all cells (the sum of HR positive and negative cells). The data are given as arithmetic means of the percentage of ASC expressing the given receptor ± SD. The numbers of volunteers from whom the data were pooled are indicated under each bar.

PBMC; 75% of IgA-ISC were found to secrete IgA1 and 25% IgA2.

3.2. KLH-Specific IgA-, IgG-, IgM-, IgA1-, and IgA2-ASC. Before immunizations, no KLH-specific ASCs were found in the circulation of any of the vaccinees. In the primed group, none of the 9 volunteers had a response after oral administration of KLH. After two subcutaneous injections, 9/9 of these volunteers had an ASC response with a geometric mean of 136 ASC/10^6 PBMC (Figure 2). In the nonprimed group, 5/5 volunteers responded after two subcutaneous KLH injections with a geometric mean of 60 ASC/10^6 PBMC (Figure 2).

In orally primed volunteers, IgA predominated in 4/9 and IgG in 5/9 volunteers. In the nonprimed group, IgA predominated in 2/5 and IgG in 3/5 volunteers.

KLH-specific IgA1- and IgA2-ASC were determined in 2/5 and 6/9 volunteers in the orally primed and nonprimed groups, respectively. The mean percentage of KLH-specific IgA1-ASC was 76% and 86% in these volunteers, respectively.

3.3. The Expression of Homing Receptors on KLH-Specific IgA-, IgG-, and IgM-ASC after Parenteral Vaccination. The expressions of $\alpha_4\beta_7$ integrin, L-selectin, and CLA on circulating KLH-specific ASC are shown in Figure 3. L-selectin was found to be expressed more frequently than $\alpha_4\beta_7$ integrin on KLH-specific ASC ($P < .001$) in both the primed ($P < .01$) and nonprimed ($P < .01$) groups. Only 35% of KLH-specific ASC expressed $\alpha_4\beta_7$ integrin in the nonprimed group and 50% in the primed group (Figure 3). L-selectin was expressed by 80% and 89% of KLH-specific plasmablasts in nonprimed and primed groups, respectively. CLA expression was determined from only three volunteers (Figure 3). The results on $\alpha_4\beta_7$ integrin- and L-selectin-expressions indicate a nonintestinal, systemic homing profile.

No major differences were seen in the homing profiles between the cells secreting IgA, IgG, and IgM (data not shown).

The sum of percentages of cells expressing the various HR exceeded 100% in all patients with data available from both $\alpha_4\beta_7$ integrin and L-selectin. The sum varied between 105.5–149.4% thus suggesting that some cells were at least double positive, that is, expressed more than one HR. This phenomenon is suggested also by the height of the columns in Figure 3.

4. Discussion

Immune responses are not evenly distributed in the body, but, instead, activated lymphocytes are guided to those tissues where their specific antigen is expected to be encountered [13]. The immune system decides on these sites on the basis of where the new antigen was first encountered; each pathogen has a typical environmental locus and route of transmission. Consequently, upon new encounters, the pathogen tends to use the same route of invasion. Thus it is advantageous to concentrate the immune response at that site. In cancer vaccines, the effector cells should be guided to the area where the cancer has originated or spread; with mucosal cancers, a mucosal homing profile, and with nonmucosal cancers, a systemic homing profile appears as to be most advantageous. The present study describes the

homing profile of the immune response to KLH, a protein carrier in numerous promising cancer vaccine candidates. These data should be applicable to all KLH-conjugated cancer vaccines. The effect of possible oral priming with KLH was explored at the same time.

Results from early clinical trials for idiotype vaccines suggested that both humoral and cellular immune responses may be independently associated with tumor regression and improved progression-free survival [7]. We have previously published data on KLH-specific T cell-mediated immunity [10, 19]; the homing profile was found to depend on the site of antigen encounter, oral or parenteral [19]. In the present study, KLH-specific B cell responses were examined in three different settings. Consistently with previous studies of B cells using the same feeding protocol for KLH [10], no response manifested by ASC was elicited by oral administration. However, in our previous studies [10, 19], it has been shown that KLH-specific T cells are found after oral KLH feeding, and, after a subsequent subcutaneous administration, the B cell response is enhanced as compared to a nonprimed group. The results of the present study appear to be consistent with those findings, as indicated by the geometric mean which appeared lower in the nonprimed than in the orally primed group (60 versus 136 ASC/10^6 cells). The enhancement of B cell response resulting from preceding oral feeding appears to offer an opportunity of utilization for cancer vaccines. However, the simultaneously induced tolerance of T cells [10, 19] appears an undesired consequence which may interfere with the idea of using oral KLH priming as a means of enhancing immune response to conjugated cancer vaccines.

In both this study and the previous studies, KLH originated from two different sources: "Calbiochem KLH" for oral use versus "Pacific Biomarine KLH" for parenteral use. Although both preparations certainly contain the same protein, they differ in the grade of purity, possibly in the assembly state of the protein, isoform proportion and percentage of denatured versus native protein. Moreover, the oral preparation was probably contaminated by endotoxin. However, these factors presumably had no major impact on the results of the present study where the immune responses were measured with a highly purified KLH preparation, and the main focus was on the homing of the KLH-specific lymphocytes.

The homing of lymphocytes into various tissues is guided by the expression of HR and CCR on their surfaces [12, 13]. The combinations of these molecules act as tracking programmes imprinted on them during activation, targeting their migration to specific tissues and microenvironments. Dendritic cells in the tissues present the antigen to the lymphocytes in the tissues, simultaneously providing instructions for the expression of HR and CCR, that is, the homing profile [12, 13]. Dendritic cells from different tissues give different instructions. The lymphocytes are generally guided to travel back to the site of antigen encounter, but possibly to certain other sites as well. Therefore, it would be useful to identify the tracking patterns so as to be able to guide the cells to sites where they are desired. Paradoxically, the majority of vaccines currently used against microbes are given by systemic route, as injections at cutaneous sites, although the majority of microbial pathogens gain access to the body through the mucosal sites. With cancer vaccines, it appears that the cells should be targeted to the site where the tumour grows and its possible metastatic foci. In the present study, a systemic homing profile with high proportions of L-selectin$^+$ ASC and low proportions of integrin $_{4\,7}^+$ ASC was revealed in both nonprimed and primed groups. However, while L-selectin expressions were identical in the two groups, the proportion of $_{4\,7}$ integrin-expressing cells appeared higher in the primed than in nonprimed volunteers. An analogous increased proportion of $_{4\,7}$ integrin in orally primed volunteers after a subsequent subcutaneous booster immunization was found in our previous study on Salmonella typhi Ty21a vaccines [28]. These data suggest a slightly increased targeting to the intestine in the primed group. This change appears undesirable for vaccines against systemic cancers and adds to the negative effects of oral priming with KLH.

The homing profile for KLH-conjugated cancer vaccines is likely to prove similar to the homing profile presented here for parenteral KLH. The vaccine was only given to healthy volunteers, no cancer patients were included. However, even if cancer patients with immunosuppression are expected to exhibit a less vigorous response, the disease will presumably have no significant impact on the homing profiles of the immune effector cells. The systemic homing profile for subcutaneous KLH indicated by the present study appears to be the desirable type of HR profile for responses against nonmucosal cancers at systemic sites, thus encouraging the development of this kind of parenterally administered vaccines.

Abbreviations

ASC: antibody-secreting cell,
CCR: chemokine receptor,
HR: homing receptor,
ISC: immunoglobulin-secreting cell,
KLH: keyhole limpet haemocyanin.

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