Clinical and Developmental Immunology

Tumor Escape and Progression under Immune Pressure

Guest Editors: Masoud H. Manjili, Nejat Egilmez, Keith L. Knutson, Senthamil R. Selvan, and Julie R. Ostberg

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Editorial Board

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Editorial

Tumor Escape and Progression under Immune Pressure

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Although cancers develop and progress in immunocompetent hosts, immunological therapies for cancer have been proposed as alternative or complementary approaches to more standard therapy. It was initially thought that tumors were silent to the immune system, and that breaking immunological tolerance could result in immune-mediated tumor rejection. However, we have learned that cancer patients have preexisting immune responses against their tumor antigens which, nevertheless, fail to protect them, in part because of increased activity of the immune suppressor cells such as myeloid-derived suppressor cells (MDSC). Attempts to develop combinatorial therapies by depleting suppressor cells or blocking suppressor pathways and at the same time actively inducing immune responses *in vivo* or adoptively transferring tumor-specific T cells have largely failed. Very limited success has been achieved only against melanoma, using adoptive T-cell therapy, or prostate cancer, using a vaccine which improves patient survival but has no apparent inhibitory effect on disease progression. Further progress in the immunotherapy of cancer has been halted because of a poor understanding of the cellular components of the immune responses working together in favor of or against the tumors, as well as our inability to reliably reprogram immune responses towards the most effective phenotypes against cancer. This special issue is focused on understanding the escape mechanisms that malignant cells develop to hijack antitumor immune responses as well as strategies to overcome tumor escape. Four main areas that are covered in this issue include the following.

Opposing Functions of the Immune System in Tumor Inhibition and Tumor Progression. Robert Schreiber proposed the term "cancer immunoediting" in order to broadly describe the dual host-protecting and tumor-sculpting actions of the immune system that not only survey for, and eliminate, nascent malignant cells but also shape neoplastic disease through equilibrium and escape mechanisms. In this issue, M. Aris et al. discuss the dual function of the immune system in controlling and promoting tumor progression in cutaneous melanoma. They propose that tumor evolution is because of a continuous feedback between tumor cells and their environment, and thus different combinatorial therapeutic approaches can be implemented according to the tumor stage. A. Amedei et al. discuss recent knowledge on the contribution of T cells in oncogenesis. They review the different types, "friend or foe," of T-cell response in gastric cancer.

Tumor-Associated Modulation of Immune Checkpoint Molecules. Upon activation, T cells develop negative feedback regulatory mechanisms in order to avoid overstimulation. These

include the expression of checkpoint molecules such as PD-1 and CTLA-4. T cells that recognize and respond to tumor antigens produce IFN-γ. A dual function of IFN-γ is the induction of apoptosis in target cells and upregulation of PD-L1 that interacts with PD-1 positive T cells, thereby resulting in the exhaustion of tumor-reactive T cells. Expression of CTLA-4 on activated T cells also results in T-cell anergy upon interaction with costimulatory molecules on DCs. S. Sapozink et al. describe new immunomodulatory approaches currently in the development pipeline, with focus on the novel CEACAM1 immune checkpoint, and compare its potential to the extensively described lymphocyte inhibitory targets, CTLA4 and PD-1. E. Rozali et al. provide an extensive review of the literature on the immunoregulatory role of PD-L2 in cancer-induced immune suppression and discuss the results of recent studies targeting PD-L2 in cancer. L. Cruz-Merino et al. discuss immune escape mechanisms in Hodgkin's lymphoma (HL) and summarize the clinical, histological, pathological, and biological factors in HL, with special emphasis on the improvement of prognosis and their impact on treatment strategies. L. Farnault et al. introduce various mechanisms involved in the escape of hematological malignancies from NK-cell surveillance. These include NK-cell qualitative and qualitative deficiencies that occur through modulating the inhibitory and activating stimuli.

Tumor-Induced Immune Suppression. Malignant cells produce cytokines and chemokines that facilitate the expansion or differentiation of immune suppressor cells such as Tregs, MDSC, and M2 macrophages. G. Zhou and H. Levitsky summarize the findings from some recent preclinical and clinical studies, focusing on how tumor cells advance their survival and expansion by hijacking therapy-induced immune effector mechanisms that would otherwise mediate their destruction. A particularly interesting notion that is touched upon involves tumor-independent treatment-induced homeostatic counter-regulation. M. Jadus et al. cover the escape mechanisms of bronchogenic lung cancer that must be overcome before they can be successfully treated. They also review the history of immunotherapy directed towards lung cancers. N. Hao et al. discuss the role of tumor-associated macrophages including M1 and M2 subsets during tumour progression and metastasis, highlighting the immunosuppressive role of M2 macrophages. V. Levina et al. investigate the role of indoleamine 2,3-dioxygenase (IDO1) in tumor escape and metastasis using 4T1 mammary carcinoma model. They show that IDO1 can not only suppress anti-tumour immune responses but also promote tumour cell proliferation.

Improved Immunotherapeutic Strategies to Overcome Tumor Escape. Immunotherapy combined with blockade of immune suppressor pathways has been developed to overcome tumor-induced immune suppression. Cornelissen et al. discuss the interplay between a dual function of the immune responses against mesothelioma which can either inhibit or stimulate tumor growth and review the challenges associated with immunotherapy. They also discuss possible strategies and opportunities to overcome tumor escape. R. Casalegno-Garduño et al. analyze the expression of the leukemia-associated antigen receptor for hyaluronan acid-mediated motility (RHAMM) in patients suffering from acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). Their results suggest that immunotherapies like peptide vaccination or adoptive transfer of RHAMM-specific T cells might improve the immune response and the clinical outcome in AML/MDS patients. S.Wallner et al. summarize the current knowledge about the negative regulatory role of Cbl-b in T-cell activation and its potential therapeutic implications for cancer immunotherapy. H. Nagai et al. demonstrate that sorafenib-induced Th1 dominance can prevent the escape of tumor cells from the host immune system in liver cirrhosis (LC) patients with advanced hepatocellular carcinoma (aHCC).

Overall, this special issue provides a well-rounded synopsis of representative research efforts addressing the issues related to "tumor escape and progression under immune pressure."

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Review Article

Role of Immune Escape Mechanisms in Hodgkin's Lymphoma Development and Progression: A Whole New World with Therapeutic Implications

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Hodgkin's lymphoma represents one of the most frequent lymphoproliferative syndromes, especially in young population. Although HL is considered one of the most curable tumors, a sizeable fraction of patients recur after successful upfront treatment or, less commonly, are primarily resistant. This work tries to summarize the data on clinical, histological, pathological, and biological factors in HL, with special emphasis on the improvement of prognosis and their impact on therapeutical strategies. The recent advances in our understanding of HL biology and immunology show that infiltrated immune cells and cytokines in the tumoral microenvironment may play different functions that seem tightly related with clinical outcomes. Strategies aimed at interfering with the crosstalk between tumoral Reed-Sternberg cells and their cellular partners have been taken into account in the development of new immunotherapies that target different cell components of HL microenvironment. This new knowledge will probably translate into a change in the antineoplastic treatments in HL in the next future and hopefully will increase the curability rates of this disease.

1. Introduction

The hallmarks of HL are mononuclear Hodgkin's cells and multinuclear Reed-Sternberg (H/RS) cells, which usually account for only 1% of cells in tumor tissue. Evidence has accumulated that H/RS cells harbor clonally rearranged and somatically mutated immunoglobulin genes, indicating their derivation, in most cases, from germinal center (GC) B cells [1–3]. Some HL cases have been identified in which the H/RS is of T-cell origin but these are rare, accounting for 1-2% of cHL. Under normal conditions, GC B cells, that lack a functional high affinity antibody, undergo apoptosis in the germinal center. H/RS cells show a characteristically defective B-cell differentiation program, lose the capacity to express immunoglobulin, and, therefore, should die. However, H/RS cells escape apoptosis and instead proliferate, giving rise to the tumor and the immune response that characterizes [1–3]. The presence of a characteristic inflammatory microenvironment is a fundamental component of the tumor mass and an essential pathogenetic factor in classical HL (cHL). It could supply the tumor cells with growth factors and could also inhibit antitumor immune responses. As the tumor cells and the reactive infiltrate grow up together, there is an extensive crosstalk between these two components mediated by cytokines and chemokines expressed by both cells. The most relevant mechanisms of immune escape are exerted by

neoplastic cells but also by specific immune cells polarized towards a Th2 phenotype in order to evade antitumor immunity. The pathogenetic role of Epstein-Barr virus (EBV) potentially based on cytotoxic T cells specifically directed towards EBV antigens also appears to influence the composition of the infiltrating immune cells population, which on the other side may have an impact on clinical presentation and outcome.

The functional role of the microenvironment and the EBV in the pathophysiology and immune escape mechanisms of HL is an exciting new field of basic and translational research. Although chemotherapy and radiotherapy remain the cornerstone of HL treatment, up to 30% and 10% of patients will recur and die of HL in advanced and early disease, respectively. Therefore, current cancer research in HL aims to develop methods to increase the effectiveness of host antitumoral immune response, mainly with biologic therapies that use the body's immune system, either directly or indirectly, to fight HL.

2. Microenvironment Composition in HL

2.1. Recruitment of HL Microenvironment. In most HL cases, H/RS cells represent the minority of the tumor burden and are dispersed among reactive elements comprising mixture of inflammatory cells, stromal cells, and a predominance of Th2 cells between the various subpopulations of lymphoid cells [4, 5]. Polarized Th1 and Th2 cells represent two subgroups of helper T cells that not only exhibit different functional properties but also show the preferential expression of some activation markers and distinct transcription factors. On the contrary to Th1 cells, the Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which are responsible for strong antibody production and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses. In such a setting, the "pressure" of the microenvironment over the neoplastic cells may be perceived as well as a strong reciprocal influence between H/RS cells and the diverse types of reactive cells. H/RS cells have a major role in the orchestration of the microenvironment milieu associated with HL. They can directly induce the recruitment of several immune cell types from the peripheral circulation and also trigger the local expansion of diverse cellular subsets. A whole plethora of soluble mediators synthesized by H/RS cells with chemotactic activity such as the cytokines and chemokines IL-5, IL-8, IL-9, CCL-5, and CCL-28 are involved in the recruitment of granulocytes, mast cells, and macrophages, whereas IL-7, CCL-5, CCL-17, CCL-20, and CCL-22 were effectors of lymphocyte recruitment and expansion [6]. Recruitment of infiltrating immune cells is also boosted by reactive cells themselves and particularly by macrophages and mast cells synthesizing CCL-3, CCL-4, and CCL-8 chemokines [6, 7].

Chemokine receptors, CXCR3, CXCR4, and CCR7, and adhesion molecules including CD62 ligand were found to be expressed on most T cells within HL tissues, while the corresponding ligands were expressed on malignant cells and vascular endothelium. These features resemble the mechanisms of T-cell recruitment observed in normal lymph nodes, thus further highlighting the crosstalk among neoplastic and nonneoplastic cells within the HL microenvironment [8].

2.2. Microenvironmental Cell Types. Innate immunity is represented essentially by dendritic cells (DCs), macrophages, natural killer (NK), NK/T cells, neutrophils, cytokines, and complement proteins, whereas adaptive immune cells are represented by B lymphocytes, $CD4^+$ T-helper lymphocytes, and $CD8^+$ cytotoxic lymphocytes (CTL). In the majority of HL tissues, different studies confirmed the predominance of $CD4^+$ T lymphocytes in the background of tumoral cells in addition to a high number of cytotoxic cells (CD8, CD57, TIA-1) (Figure 1) [9–11]. The composition of the infiltrate has been seen to differ depending on the histological subtypes of cHL and the discrete stages of the disease course but also on the state of immunosuppression of HL patients. The reactive background is most pleomorphous in HL cases of the mixed cellularity histotype (MCHL), where inflammatory elements efface the lymph node architecture, while it is mainly composed of lymphocytes organized within preserved or regressed lymphoid follicles in cases of the lymphocyte-rich type (LRHL) [12–17]. In the nodular sclerosis variant (NSHL), the presence of a prominent mixed inflammatory background may be progressively reduced by the accumulation of collagen fibrosis suggesting a dynamic process of tissue remodeling [12–17].

2.3. Crosstalk between Tumoral and Immune Cells. The continuous interaction pathways of H/RS cells with nonmalignant reactive and stromal cells in lymphoma tissues is now a clear evidence [6]. Several observations indicate that H/RS cells are dependent on survival signals received from immune/inflammatory cells [18]. $CD4^+$ T cells, the largest population of infiltrating immune cells, are presumably particularly important [19]. Some of the survival signals that are provided by inflammatory cells to the H/RS cells are the triggering of CD40 signaling by CD40L-expressing rosetting T cells, activation of TACI and BCMA through production of their ligand APRIL by neutrophils, and perhaps activation of CD30 through CD30L-expressing mast cells and eosinophils. Moreover, H/RS cells express IL-3R, which has growth- and survival-promoting effects following activation, and there is evidence that H/RS cells can induce activated T cells to secrete IL-3 [20]. H/RS cells stimulate fibroblasts through various factors (e.g., TNFα, transforming growth factor-β (TGF-β), and fibroblast growth factors) [21, 22], and the activated fibroblasts in turn produce eotaxin and CCL5, thus contributing to the attraction of eosinophils and Tregs into the lymphoma [22]. H/RS cells also orchestrate their cellular microenvironment to evade an attack by cytotoxic T cells or natural killer cells. The presence of a large population of Tregs in the HL microenvironment is presumably established not only by the chemokine-mediated attraction of such cells but also by induction of differentiation of naive $CD4^+$ T cells into Treg cells by H/RS cells [23]. Unexpectedly, high numbers of Treg cells in the HL microenvironment have been

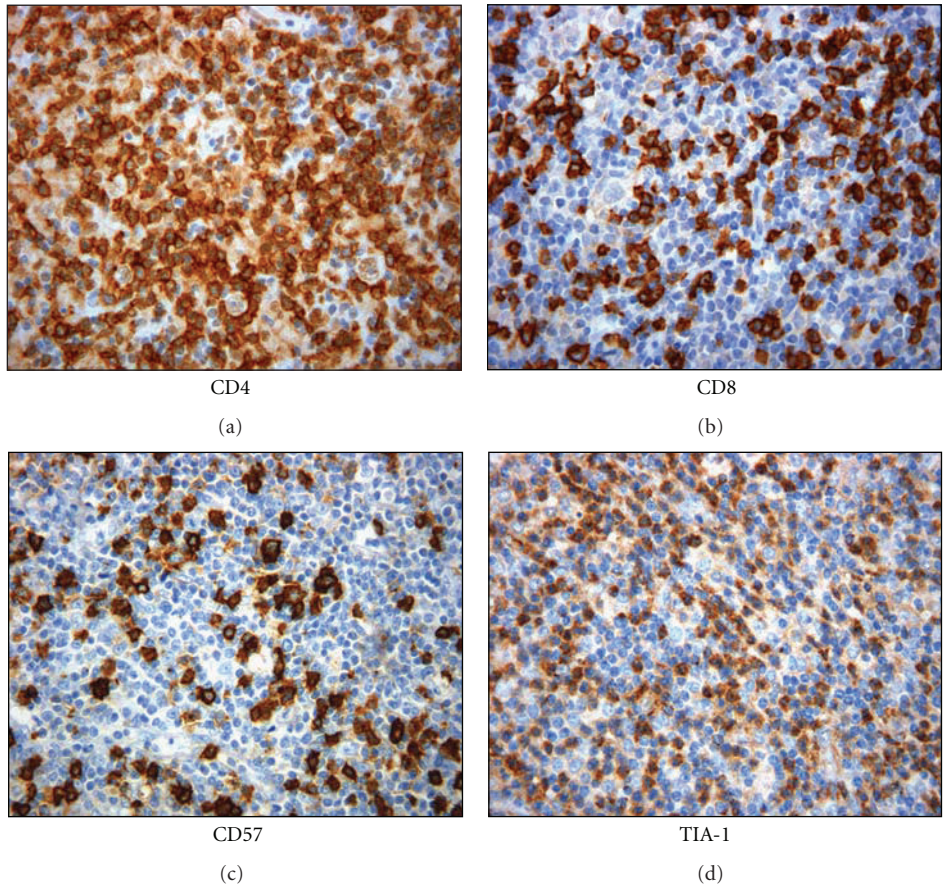
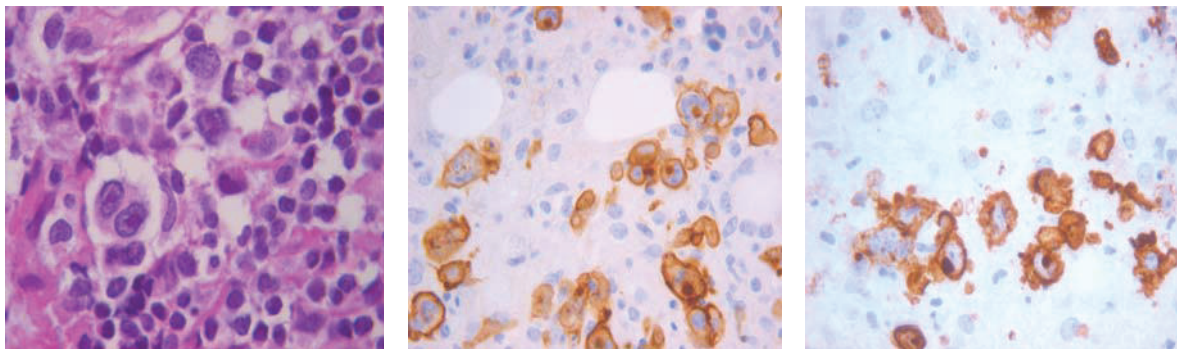


Figure 1: Immunohistochemical staining of inflammatory background in HL: T lymphocytes (CD4 and CD8), NK cells (CD57), and cytotoxic cells (TIA-1).

linked to a good prognosis, indicating that Treg cells may also have some suppressive activity on H/RS cells or on other inflammatory cells that support H/RS cell survival and/or proliferation [24, 25]. H/RS cells may further modulate their cellular microenvironment by shifting a Th1-type response to a Th2 response, which often has tumor-promoting activities [26]. H/RS cells also produce the immunosuppressive cytokines IL-10 and TGF-β, and galectin 1 (Gal-1) and prostaglandin E2, which inhibit T-cell effectors functions [27–31]. Moreover, T-cell effectors functions are inhibited by binding of programmed cell death protein 1 (PD-1) on T cells to the PD1 ligand that is expressed by H/RS cells [32, 33].

2.4. Microenvironment, Hematopoiesis, and Extracellular Matrix. The hematopoietic microenvironment is constituted by a three-dimensional complex and highly organized structure (stromal cells, extracellular matrix (ECM), and cytokines/chemokines), which serves to regulate the location, proliferation, and function of the hematopoietic cells [34]. Their alterations not only have great importance in the physiopathology of some leukemia/lymphoma but also in the formation of the intratumoral cell microenvironment. ECM represents a biophysical filter that offers protection, nutrition, and cell innervation, giving way for immune response, angiogenesis, fibrosis, and tissular regeneration [35]. Its disruption supposes a functional loss for nutrition, elimination, cell denervation, regenerative capacity, and wound healing. This also causes the loss of the immune response to pathogens, toxins, and tumoral cells. HL was the first hematopoietic tumor to be characterized as having clearly aberrant nuclear factor-κB (NF-κB) activity that appears closely linked to the cellular interactions within the bone marrow microenvironment: direct contact of tumoral cells with the EMC, bone marrow stromal cells (BMSCs), osteoblasts, or other cellular compartments in the BM. In HL, NF-κB is constitutively activated and serves as survival factor of tumoral cells [36–38]. To date, several pathways have been suggested to induce aberrant signaling in H/RS cells, including expression of Epstein-Barr virus latent membrane protein-1 (EBV-LMP-1), increased IKK activity, functional expression of receptor activator of NF-κB (RANK), or ligand-independent signaling following overexpression of CD30 [39, 40]. EBV, similarly to other viruses and certain bacteria, may induce pathological changes by epigenetic reprogramming of host cells. In HL, LMP1 can modulate cellular gene expression programs by affecting, via the NF-κB pathway, levels of cellular microRNAs miR-146a and miR-155 [41]. Elucidation of the epigenetic consequences of



(a) (b) (c)

Figure 2: Reed-Sternberg cell (a) seen in a cellular background rich in lymphocytes of a classical Hodgkin's lymphoma. Immunohistochemical expression of the activation markers CD30 (b) and CD15 (c).

EBV-host interactions (within the framework of the emerging new field of pathoepigenetics) may have important implications for therapy and disease prevention, because epigenetic processes are reversible, and continuous silencing of EBV genes contributing to pathoepigenetic changes may prevent disease development.

3. Hodgkin's Lymphoma in Immunosuppressed Patients

cHL in non-immunosuppressed and immunosuppressed individuals are similar in morphology of neoplastic cells, expression of activation markers such as CD30 and CD15 (Figure 2), and aberrations/activation of NF-κB pathway, but they differ in the strict association with EBV infection, persistent B-cell phenotype, and CD4 cellular background composition [42]. In immunosuppressed hosts, according to the type of immunosuppression, cHL include human-immunodeficiency-virus- (HIV-) associated, iatrogenic, and posttransplant types.

3.1. HIV-Associated cHL. cHL represents the most common nonacquired immunodeficiency syndrome (AIDS) tumor diagnosed in patients with HIV infection. The risk is significantly increased in all ages and the risk relative to the general population ranging from 5- to 15-fold [19, 43–45]. Moreover, a significant increase in the incidence of cHL in patients treated with highly active antiretroviral therapy (HAART) has been observed. HIV-HL exhibits pathological features that are different from those of HL in "general population" with predominance of unfavorable histological subtypes (MC and LD) [46–48]. One of the peculiar clinical features of HIV-cHL is the widespread extent of the disease at presentation and the frequency of systemic B symptoms [48]. The widespread use of HAART has resulted in substantial improvement in the survival of patients with HIV infection and lymphomas because of the reduction of the incidence of opportunistic infections, the opportunity to allow more aggressive chemotherapy, and the less-aggressive presentation of lymphoma in patients in HAART compared with those lymphomas which arise in patients who never received HAART [49, 50]. Optimal therapy for HIV-cHL has not been defined yet. The widespread use of HAART allows the use of more aggressive chemotherapeutic regimens generally used in cHL in HIV negative patients. Since a large proportion of HIV-cHL progresses and relapses, the use of HDC and autologous stem cell transplantation (ASCT) has been tested in this setting [51–53].

3.2. Posttransplant Associated cHL. The majority of transplanted patients are initially managed by reduction and/or withdrawal of immunosuppression. Posttransplant lymphoproliferative disorders (PTLDs) are a heterogeneous group of monoclonal or polyclonal lymphoproliferative lesions that occur in immunosuppressed recipients after solid-organ or bone marrow transplantation [54, 55]. Generally, the time from transplant to the development of the disease ranges from few months (4–6 months) to several years, with a median time of 113 months, significantly longer than that of classical B-cell PTLDs. The posttransplant cHL setting, most often in renal transplant patients, is almost always EBV positive [56–59]. The distinction of Hodgkin's-like PTLD from true Hodgkin-type PTLD may be difficult [56]. Different studies have described the clinical course, generally very aggressive, and the poor outcome of patients receiving posttransplant immunosuppression. The use of chemotherapy is limited by the clinical condition of patients, and the response rate is generally lower than that observed in all other forms of cHL. Recently, rituximab has also gained favor in the treatment of PTLD because of its targeting of CD20-positive B cells, with fairly promising results [60].

3.3. Refractory cHL. Refractory cHL patients are defined as patients who do not respond to first-line chemotherapy or progress during treatment or relapse within 3 months after the first-line therapy. They represent 20–25% of cHL with advanced stage disease [61, 62]. Many of these patients have a poor overall survival and may die as result of their disease. To date, there is no consensus on biological markers that add value to usual parameters (which comprise the IPS) used at diagnosis to predict outcome. The prognostic significance of CD20 expression in cHL is controversial and a matter of

Table 1: Recompilation of the different factors implicated in the tumoral immune escape in HL.

Strategy	Mechanisms	Regulated factors
Tumoral protective action	Upregulation of growth and survival receptors expression [6]	IL-7R, IL-9R, IL-13R, TACI, and CCR5 tumoral cells
	Downregulation of transcription factors [71]	IL-6R, TACI, RANK, TNFR-1, Cys-LT receptors, and NOTCH-1
	Upregulation of Th2 cells attractant chemokines [28]	TARC, MDC
	Upregulation of apoptosis/proliferation modulators [20, 28, 72–74]	Fas, FasL, IL-1β, TGF-β, TNFR, IL-13, IL-3
	Upregulation of immunoregulatory protein and regulatory T cells [27, 33]	Gal-1, PD1
	Downregulation of adhesion factors [79]	HGF, c-MET
	Downregulation of cytotoxic cells activity [75, 77, 126–135]	MHC I, PI9, IL-10, TGF-β, LAG-3, CTLA-4
	Upregulation of inhibitory T cells activator [31]	PGE2
	Selection of minor side population [87, 88, 91]	MDR1, ABCG2, gemcitabine resistance factor
Reprogramming of tumoral cells	Mutations/downregulation of MHC class II [92]	CIITA
	Upregulation of death receptors ligands [33]	PDL1
	Upregulation of immunosuppressive factors [32, 93]	TGF-β, PD-1
Tolerance induction by TAM	Macrophage deviation to Th2 differentiation (TAM) [110, 112, 113]	IL-6, TNF, IL-1β, IL-23
	Upregulation of inflammatory and matrix-remodeling genes [125]	C1Qalpha, C1Qbeta, and CXCL9
Tolerance induction by Tregs	Conversion of naïve regulatory T cells to $CD4^+$ $CD25^+$ [101, 102]	Foxp3
	Dowregulation of CTL activity [104, 105]	IL-2Rα (CD25), Il-10, TNF-β

ongoing debate [63–66]. A recent retrospective study shows that new immunohistochemical markers might predict the response to treatment of cHL based both on features of tumoral cells and on microenvironment [67]. Patients presenting either a refractory and early relapse cHL or a responding disease provided evidence that HRS cells present at diagnosis an overexpression of BCL2 marker and a frequent absence of CD20 expression and that there is an excess of cytotoxic TIA-1 and ckit-positive mast cells in the microenvironment. In patients with refractory disease, who have attained at least a partial response after salvage therapy, intensification with high-dose chemotherapy (HDC) significantly improves the outcome of patients. However, patients with primary refractory disease still showed a worse prognosis [68, 69].

4. Immune Escape Mechanisms in HL and Prognosis

The immune system has the ability to act as a double-edged sword, indicating that tumor elimination requires a good coordination of the various elements of the immune system (Table 1). If tumoral cells employ a plethora of immunosuppressive mechanisms, which may act in concert to counteract effective immune responses, different subsets of immune cells contribute also to this immunosuppressive network (Figure 3) [70].

4.1. Tumoral Protective Action. The microenvironment of HL is sustained by an autocrine and/or paracrine production of several cytokines including, among others, IL-5, IL-8, IL-9, CCL-5, and CCL-28. The release of these molecules is also responsible for most of the symptoms recorded in patients with HL, in addition to the ability of the neoplastic cells to escape from growth controls and immunosurveillance. Effectively, H/RS cells are able to sense growth and survival signals coming from the growth factor milieu, owing to the expression of a broad range of receptors including IL-7R, IL-9R, IL-13R, TACI, and CCR5 [6]. Along with growth factors, also proinflammatory cytokines and mediators can sustain H/RS cell expansion through the activation of pathways converging into the NF-κB focal point, such as those triggered by IL-6R, TACI, RANK, TNFR-1, Cys-LT receptors, and NOTCH-1 engagement [71]. These proinflammatory spurs may be either derived from the microenvironment (e.g., leukotriene production by mast cells and NOTCH-1 ligand expression by stromal cells) or originate from both H/RS cells and reactive elements (e.g., IL-6, TNF).

The H/RS cells secrete high amounts of chemokine, thymus and activation-regulated chemokine (TARC) and macrophages-derived chemokine (MDC) in particular, which attract lymphocytes expressing CCR4 receptor, such as Th2 [28]. These cytokines may contribute to the pathogenesis of the disease initiated and sustained the presence of the reactive infiltrate. Immune cells present in the local infiltrate have proved to be capable of modulating apoptosis and of

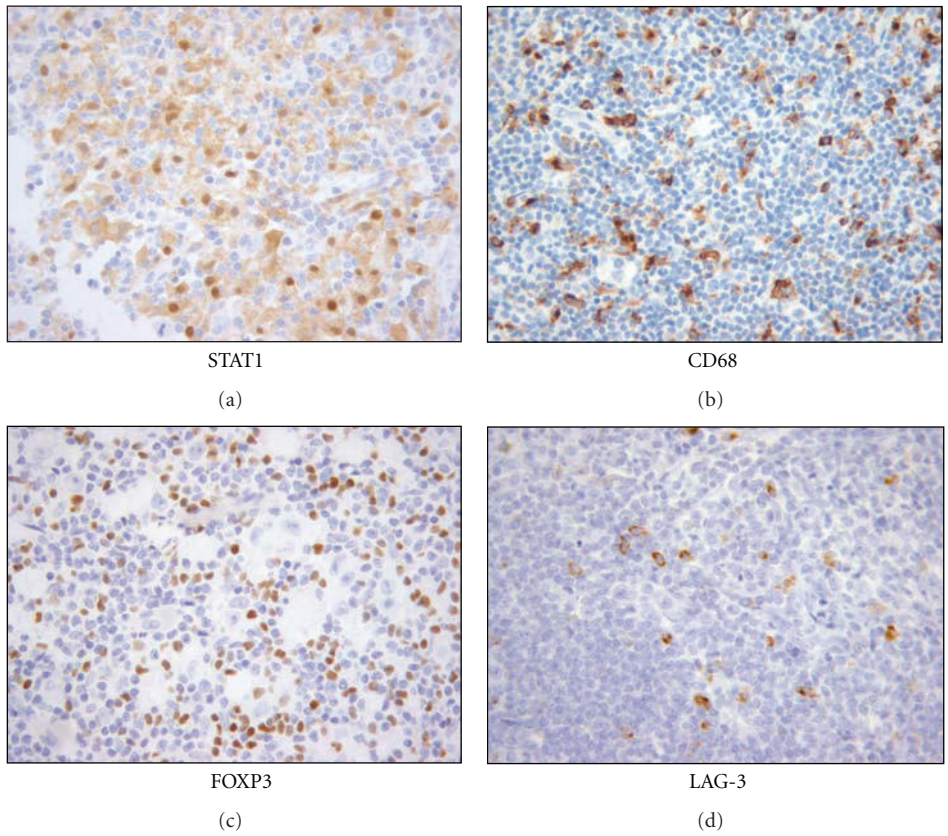


Figure 3: Immunohistochemical staining of immunosuppressive cells in HL: tumor-associated macrophages TAM (STAT-1 and CD68) and regulatory T cells (FOXP3 and LAG-3).

inducing proliferation of tumoral cells via death receptors, cytotoxic granule liberation, and withdrawal of growth factors or production of immunosuppressive cytokines [28, 72–74]. In HL, it has been initially proposed that $CD4^+$ T cells produce cytokines of Th2 type that could contribute to local suppression of the cellular immune response mediated by Th1 cells [75, 76]. Immunoregulatory cytokines such as IL-10 and TGF-β play an important role in immune tolerance, and it seems that suppressor effect of regulatory T cells ($CD4^+CD25^+$) on the development of tumor associated antigen-reactive lymphocytes is independent of cytokines [20].

Several other molecules have been tested for their possible involvement in such a context. For instance, Prostaglandin E2 has been shown to impair $CD4^+$ T-cell activation [31]. Tissue inhibitor of metalloproteinases 1 (TIMP-1) is a protein with proteinase-inhibiting and cytokine properties which has been advocated not only as a survival factor for H/RS cells but as potential immunosuppressive agent. Also, the downregulatory molecule cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) was shown to play a possible role, as the proportion of $CTLA\text{-}4^+/CD3^+$ cells negatively correlated with proliferative activity, IL-2 and IFN-γ production by T lymphocytes in HL patients [77]. Even CD30, which is typically expressed on HRSC, was shown to inhibit T cell proliferation [78]. Other potentially involved molecule is the Gal-1, produced by H/RS cells. In fact, blockade of Gal-1 was able to restore the Th1/Th2 balance [27]. It has been also proposed that hepatocyte growth factor and c-MET might constitute an additional signaling pathway between H/RS cells and the reactive cellular background, affecting adhesion, proliferation, and the survival of H/RS cells [79].

The 15–25% of HL patients who did no respond to standard chemotherapy regimens will die of relapse [80], probably due to the presence of a small number of cells resistant to chemotherapy or radiation treatment that are not eliminated by the endogenous immune system. This minor side population (SP) of tumor cells has been previously identified as cells with stem/progenitor cell-like characteristics from normal [81, 82] and malignant tissues [83–86]. These SP cells also express multidrug transporter proteins, including MDR1 and ABCG2 [87, 88], which not only efflux Hoechst dye but also rapidly reduce the intracellular concentrations and thus the cytotoxicity of many commonly used therapeutic drugs [89, 90]. In a more recent study, Schafer and collaborators identified a distinct SP subset in HL cell lines and primary tumor biopsies that are resistant to gemcitabine [91]. This SP subset also expresses tumor-associated antigens, which render them susceptible to killing by tumor-specific CTLs following demethylation with decitabine. This study suggests that combination therapeutic strategies that use conventional chemotherapy to debulk tumor burden,

followed by novel drugs such as histone deacetylation (HDAC) inhibitors and T cell immunotherapy, may eliminate residual chemoresistant tumor cells and help prevent disease relapse.

4.2. Reprogramming of Tumoral Cells. The escape from apoptosis and transcriptional reprogramming of H/RS cells are interlinked and seem important to disease pathogenesis. In cHL and primary mediastinal B-cell lymphoma, genomic breaks of the major histocompatibility complex (MHC) class II transactivator CIITA have been demonstrated to be highly recurrent (15% and 38%, resp.) [92]. The functional consequences of CIITA gen fusions is the downregulation of surface HLA class II expression and overexpression of ligands of the receptor molecule PD-1 (CD274/PDL1 and CD273/PDL2). These receptor-ligand interactions have been shown to impact antitumor immune responses in several cancers, whereas decreased MHC class II expression has been linked to reduced tumour cell immunogenicity. The exploration of the possible role played by the PD-1 protein shows that this molecule (expressed on the surface of activated T cells, B cells, and macrophages) and its ligands are involved in the functional impairment of T cells in chronic viral infections or HL tumor immune evasion. HL was shown to overexpress PD-1 ligand, while PD-1 was markedly elevated in tumor-infiltrating and peripheral T cells of these patients. Moreover, blockade of the PD-1 system was able to restore the IFN-γ production by HL-infiltrating T cells [33]. Using a genome-wide transcriptional approach, $CD4^+$ T cells in HL were demonstrated to be under the inhibitory influence of both TGF-β and PD-1 in vivo [32]. An increase in the number of PD-1^+ lymphocytes, measured within a tissue microarray platform, was also shown to be a stage-independent negative prognostic factor of overall survival as opposed to the number of $FOXP3^+$ Tregs [93]. All these findings seem to suggest that the impairment of the typical immune response in HL, is, at least partially, mediated by the PD-1 signaling pathway.

4.3. Regulatory T Cells (Tregs). The categorization of $CD4^+$ T cells in Th1 and/or Th2 constitutes an oversimplification and it has been shown that regulatory T cells with $CD4^+CD25^+$ phenotype not only play a role in controlling autoimmunity but also have suppressive effects on immune responses [94–96]. In cancer-bearing animals or patients, Tregs expand, migrate to tumor sites, and suppress antitumor immune response mediated by NK cells, $CD4^+$ and $CD8^+$ T cells, and myeloid cells, through different molecular mechanisms [97]. Functional and molecular characterization of these cells has been facilitated by the identification of markers such as FOXP3 and others [98–100]. FOXP3 encodes a transcription factor known as Scurfina, specifically expressed by T cells $CD4^+CD25^+$ [101], that acts on converting naïve regulatory T cells $CD4^+CD25^-$ phenotype to $CD25^+$ [102]. More recently, it was suggested that regulatory T cells and $PD1^+$ T cells interact with H/RS cells [24, 33, 103], which produce the T regulatory attractant Gal-1 and the PD-1 ligand, PDL-1 [33]. On the other hand, the observation of numerous $CXCR3^+$ lymphocytes in some HL tumors has raised the possibility of an occasional Th1-predominant immune response [10].

The regulatory T cells can inhibit the production of IL-2 to regulate the high expression of IL-2Rα (CD25), that is, delay or block the activation of $CD8^+$ cells and NK cells against tumor antigens [104, 105]. The immunosuppressive properties of regulatory T cells appear to be particularly important because of its large effect on cellular cytotoxicity represented by CTLs and NK cells. The presence of low numbers of $FOXP3^+$ cells and a consequent high rate of TIA-1^+ cells in the infiltrate represents an independent prognostic factor negatively affecting the survival of the disease. Furthermore, when the disease relapses and progresses, larger number of TIA-1^+ cells and lower proportion of $FOXP3^+$ on the reactive background of the tumor are also prone to be seen [24].

It has been also hypothesized that the contribution of Tregs to HL might be function of the microenvironment polarization. Indeed, Tregs may limit the inflammatory spur of other cells of the immune system (including T effectors) by releasing IL-10 and TGF-β, and this beneficial effect may prevail over the impairment of an effective T-cell-mediated response, as far as the outcome of HL is concerned. Nevertheless, when the HL-associated environment is diverted towards marked inflammation owing to the abundant presence of mast cells and macrophages, the regulatory function of Tregs may prove inadequate to restore the balance between pro- and antiinflammatory stimuli, and Tregs can even boost inflammation through TGF-β release and Th17 generation. Under these circumstances, a direct role for mast cells in the Tregs contrasuppression and Th17 deflection can be envisaged as both mast cells and Tregs populate HL infiltrated areas, and their interaction is therefore possible. In an effort to inhibit suppressive signals counteracting activation, removal of Tregs leads to effective antitumor immunity [106]. In certain solid tumor models, depletion of Tregs in combination with immunostimulatory treatments even causes rejection of already established tumors [99, 107].

4.4. Tumor-Associated Macrophages (TAM) and Myeloid-Derived Suppressor Cells (MDSC). Chronic inflammation in some tissues correlates with higher risk of developing tumors [108]. Within the tumor microenvironment, tumor-associated macrophages (TAM) and myeloid-derived suppressive cells (MDSC) seem to play a critical role in the progression of tumor development through nonimmune (mostly proangiogenic) and immune mechanisms [109]. TAMs are a heterogeneous population of cells depending on oxygen availability and phases of tumor development [110]. In early stages, tumors are generally infiltrated by type 1 macrophages (M1) that release proinflammatory cytokines and chemokines promoting Th17 cell differentiation from naïve $CD4^+$ T cells [111]. On the other hand, in advanced stages, TAM polarize to a type-2-macrophage- (M2-) related cell that releases cytokines such as TGF-β1 and IL-10, which induce Th2 differentiation and recruitment, favoring Tregs development and thus promoting tumor development

through inhibition of anticancer immune responses [112]. It is now accepted that TAM, major players in the connection between inflammation and cancer, summarize a number of functions (e.g., promotion of tumor cell proliferation and angiogenesis, incessant matrix turnover, repression of adaptive immunity) which ultimately have an important impact on disease progression [113–115]. High levels of TAM are often, although not always, correlated with a bad prognosis, and recent studies have also highlighted a link between their abundance and the process of metastasis [116–119]. Macrophage infiltration began very early during the preinvasive stage of disease and increased progressively [120]. This pathological evidence has been confirmed also at gene level, where molecular signatures associated with poor prognosis in lymphomas and breast carcinomas include genes characteristic of macrophages (e.g., CD68) [121–123]. In human HL progression, macrophages are anything but innocent bystanders since the expression of CD68 showed to be the best predictive biomarker for risk stratification and survival for this type of cancer. A high number of $CD68^+$ cells correlates with primary and secondary treatment failure [124]. Another report suggested that TAMs in HL subtypes might differ in their expression of inflammatory and matrix-remodeling genes [125].

Recent studies in model animal suggest that macrophages were responsible for restoring tumor vascularisation and repair after irradiation [136]. In effect, after irradiation, the remaining tumor mass sends tissue damage signals to initiate repair, which includes the recruitment of macrophages to the tumor, aiding in the recovery of tumor growth by enhancing angiogenesis, supplying growth factors, and creating a local immunosuppressive environment. Treatment of tumors with antibodies specific for CD11b can block macrophages recruitment and inhibit tumor regrowth and survival [137]. In the same way, the use of clodronate, a liposome toxic to phagocytes, or the use of Enbrel, a blocking against TNF-α, inhibits tumor recovery after irradiation [136]. The detrimental contribution of mast cells and $CD68^+$ macrophages to HL patients' survival has been clearly established and has been linked to the ability of both types of cells to induce and maintain the aforementioned proinflammatory microenvironment [124, 138, 139].

4.5. Cytotoxic T-Cells Inhibition. Different mechanisms have been suggested to account for the CTL-mediated apoptosis resistance of H/RS cells, such as the downregulation of MHC class I molecules of the H/RS cells, prevention of recognition of tumor-associated antigens by CTLs [126], or the local secretion of both IL-10 and TGF-β by H/RS cells [127, 128], which are able to inhibit CTL function. In this respect, it appears that the blockage of the Granzyme B pathway of apoptosis through the overexpression of serine protease inhibitor PI-9/SPI-6 is an important additional mechanism for immune escape by tumors [129]. The expression of PI9 tends to be associated with a high percentage of activated CTLs, especially in HL [75], explaining why tumors expressing high levels of PI9 have a particularly poor clinical outcome.

LAG-3 was found strongly expressed on Tregs present in the proximity of H/RS cells and the proportion of LAG-3-expressing lymphocytes correlated with the EBV status of the tumor [130]. The level of LAG-3 expression on the Tregs was coincident with impairment of LMP1/2-specific T-cell function [130] suggesting a pivotal role for $LAG\text{-}3^+$ regulatory T cells in the suppression of EBV specific cytotoxic $CD8^+$ cell-mediated immunity in HL [130]. It has been suggested that LMP1- and EBNA1-specific HLA class II-restricted peptide epitopes can selectively recruit regulatory T cells and impair antigen-induced IFN-γ production [131, 132]. LAG-3 has high affinity for MHC class II molecules and downregulates CD3 T-cell receptor mediated signaling and blockade of LAG-3 mediated signaling induces enhanced activation of human CD8 T cells [133–135]. Preliminary results of EBV specific CTL therapy in relapsed/refractory EBV-positive HL patients are encouraging [140] and, taken together, these findings have important implications in the improved design of immunotherapeutic strategies to boost LMP1/2-specific CTL activity.

5. New Molecular Prognostic Parameters versus Traditional Clinicobiological Prognostic Parameters

A huge amount of clinical and biological factors have been related with the risk of relapse and progression in HL, and consequently with the therapeutical strategy planned in every single patient. Recent contributions determine that HL represents the prototypical tumor in which the interplay between H/RS and the reactive microenvironment determines not only the histological morphology and classification but also the clinicopathological features and prognosis of these patients [141].

5.1. Traditional Clinicobiological Parameters. Similar to other lymphomas, nowadays selection of treatments in HL continues to depend on initial risk stratification. In this sense, stage remains the single most important factor in the initial approach for treatment of HL, being the Ann Arbor's system with Cotswolds modifications the current staging system used for patients with HL [142].

In clinical practice, HL is classified in early and advanced disease [143]. Early disease includes stages I-II and it is generally divided into favorable and unfavorable categories based upon the presence or absence of certain clinical features, such as age, erythrocyte sedimentation rate (ESR), B symptoms, and large mediastinal adenopathy. Cooperative research groups have used diverse definitions of favorable and unfavorable prognosis disease [144]. This stratification is highly pertinent and useful since patients with favorable prognosis disease may have acceptable outcomes with less intensive therapy than that required for those with unfavorable prognosis early stage or advanced stage disease [145].

Among patients with advanced stage HL (stage III/IV, and for some groups stage II plus bulky nodal disease), prognosis is largely determined by the International Prognostic Score (IPS) [146]. The IPS was created by the IPS Project on

Advanced Hodgkin's Disease after analyzing several possible prognostic factors in 1,618 patients that were treated mainly with ABVD-like regimens. Finally, the IPS is based upon the total number of seven potential unfavorable features at diagnosis: serum albumin less than 4 g/dL, hemoglobin less than 10.5 g/dL, male gender, age over 45 years, stage IV disease, white blood cell count ≥15,000/microL, and lymphocyte count less than 600/microL and/or less than 8 percent of the white blood cell count. All of these adverse prognostic factors were statistically significant at the multivariate analysis. Patients with four or more adverse features had a significantly inferior freedom from progression (47% versus 70%) and overall survival (59% versus 83%). Coupled with stage, the IPS allows identification of a poor-risk group of patients requiring more intensive therapy [146]. Consequently, different treatment policies are indicated upon the presence of these clinicobiological parameters, with application of more aggressive approaches when more risk factors are present.

5.2. Innovative Biologic Prognostic Parameters. Current predictive systems, determined by clinical and analytical parameters, fail to identify high-risk patients accurately (patients who relapse or die). Quantitative analysis of infiltrating immune cells reveals undisclosed relationships between the relative proportion of these cells and HL clinical outcome, illustrating how factors other than tumoral cellularity, or the immunophenotype and molecular anomalies present in the H/RS cells, can play a role in tumoral behavior. Regardless of the classic clinical and pathological features, a high proportion of infiltrating $CD8^+$ and $CD57^+$ cells as well as a low number of infiltrating CTL (evaluated by the presence of Granzyme B and TIA-1) appear to be associated with a favorable outcome for HL patients (without B symptoms and lower clinical stages) and better response to treatment [10, 24, 147]. It is unclear to date whether the presence of $CD8^+$ T cells correlates with the antitumor cytotoxic response. Nevertheless, it has been suspected that $CD8^+$ T cells may be recruited in an antigen-nonspecific mode in HL (Figure 4) [148].

A multistep approach to design a quantitative PCR assay has been applied to routine formalin-fixed paraffin-embedded sample integrated genes expressed by the tumor and their microenvironment [149]. In cHL with advanced stage, specific gene signatures associated with favorable or unfavorable clinical outcome have been identified. The best predictor genes were integrated into an 11-gene model, including 4 functional pathways: cell cycle (CCNA2, CDC2, HMMR, CCNE2, CENPF), apoptosis (BCL2, BCL2L1, CASP3), macrophage activation (LYZ, STAT1), and interferon regulatory factor 4. These genes are able to identify low- and high-risk patients with different rates of 5-year failure-free survival: 74% versus 44.1% in the estimation set and 67.5% versus 45.0% in the validation set.

Although the activation status of infiltrating cells have been demonstrated to be independent of the degree of malignancy in HL [150], others studies have shown that the presence of activated cytotoxic T cells (granzyme B^+) is associated with unfavorable followup in these patients [11, 151, 152]. A higher level of not activated cytotoxic cells (TIA-1^+) has been observed in advanced-stage cHL without prognostic value [153]. However, TIA-1^+ CTL associated with the presence of regulatory T cells $FOXP3^+$ appears to play an important role in monitoring HL patients [24]. Variations in the level of killer cells and TIA-1^+ regulatory T cells observed during the course of the disease could be implicated in the progression of HL [24].

Association of tumor-associated macrophages (TAM) $CD68^+$ with adverse clinical outcomes has been confirmed in several studies in hematologic and solid tumors [139]. Recently, a gene expression profile analysis performed on 130 biopsy samples from HL patients identified a signature of TAM and monocytes that was predictive of treatment failure [124]. When compared with those with low CD68 expression, patients with tumors that demonstrated an increased number of CD68 expressing macrophages had shorter median progression-free survival (PFS), lower rate of 10-year disease-specific survival (60 versus 89%), and higher failure rate of secondary treatment with curative intent (63 versus 13%). It has been also recently demonstrated that high level of CD68 correlated with poorer survival, event-free survival, and with the presence of EBV in the tumor cell population [154].

Biologic markers associated with apoptosis/proliferation have been also studied in HL. Different studies have described alterations in genes controlling apoptosis and proliferation of H/RS cells and biological factors such as EBV detection, which influence the clinical aggressiveness of the disease [155–165]. Shorter survival was significantly associated with high proliferation index (Ki67), high expression of bcl2, bcl-xl, bax and p53, low expression of Rb and caspase 3, and high apoptotic index [163, 166–173]. Evidence has accumulated that the constitutive activation of the NF-κB pathway in H/RS cells is of particular importance for explaining the apoptosis deregulation in cHL [157, 158, 160, 174]. By gene expression profiling, the good outcome cHL was characterized by upregulation of genes involved in apoptosis induction and cell signaling, including cytokines and transduction molecules, while the bad outcome cHL were characterized by upregulation of genes involved in cell proliferation (Ki67) and by downregulation of tumor suppressor genes PTEN (phosphatase and tensin homolog deleted on chromosome 10) and DCC (deleted in colorectal cancer) [175].

Immune cells present in the infiltrate have been shown to modulate the apoptosis and proliferation of tumor cells via apoptotic receptors, cytotoxic granule release, growth factors, or immunosuppressive cytokines [72–74, 176, 177]. IHC study has demonstrated that the antiapoptotic profile observed in H/RS cells is associated with a general increase in $CD4^+$ T cells infiltrating (related to Bcl-XL and Mcl-1) and an overall decline $CD8^+$ T lymphocytes infiltrating, NK cells, and dendritic cells (related to Bcl-XL and Bax) [178]. The infiltrated immune cells are able to activate apoptotic caspase proteolytic cascade through TNF receptor superfamily interactions (FasL/Fas and CD40/CD40L) [158, 179–183]. CTLs are also able to trigger a second proapoptotic pathway

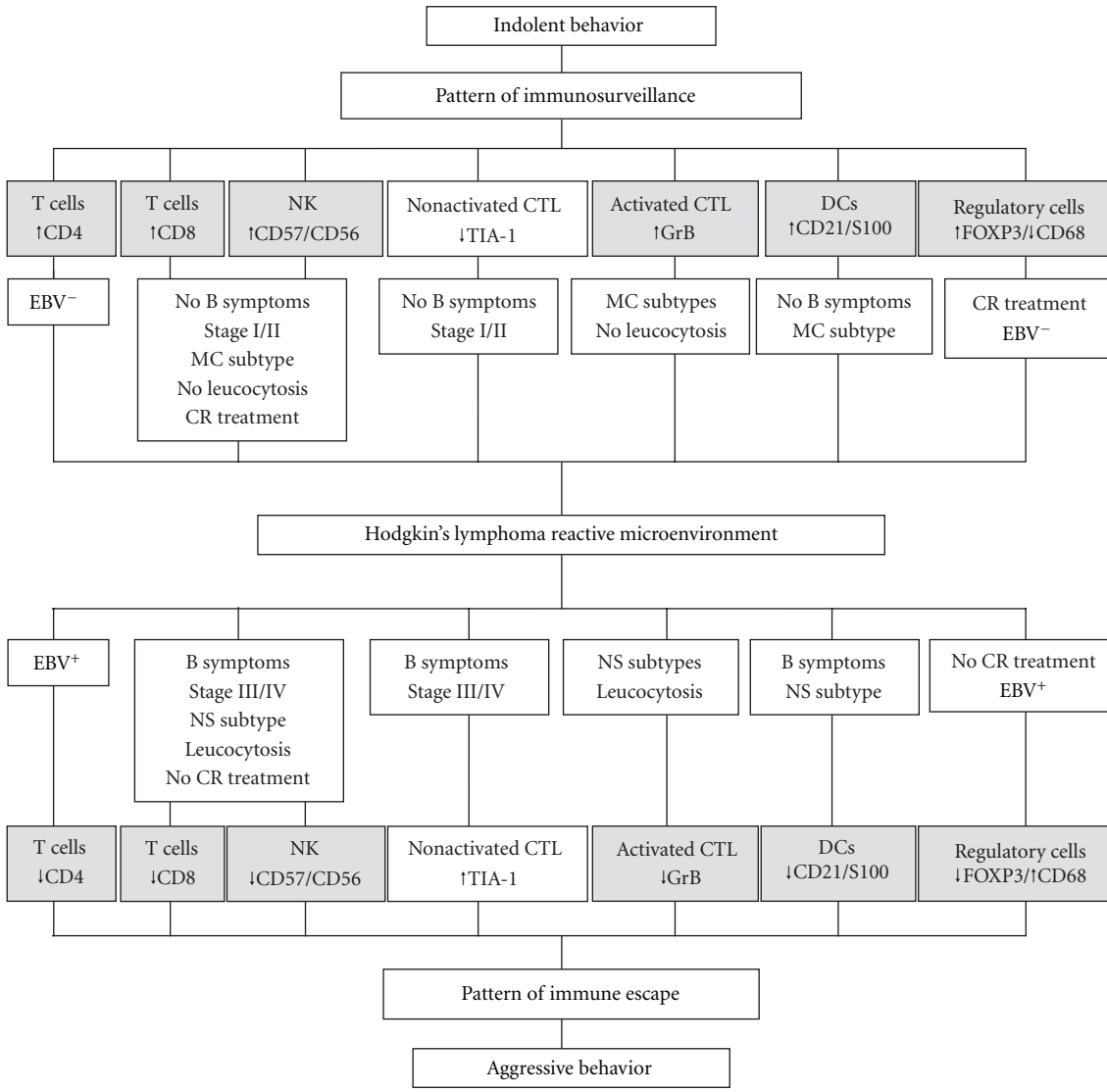


Figure 4: Representation of the two immune patterns observed in HL significantly associated with their clinicopathological features. The immunosurveillance pattern with a high proportion of infiltrating T lymphocytes, NK cells, DCs, activated CTL, but low proportion of resting CTL and TAM is associated with a favorable outcome. The immune escape pattern with a high proportion of infiltrating resting CTL and TAM, but low proportion of T lymphocytes, NK cells, DCs, and activated CTL is associated with an unfavorable outcome. MC, mixed cellularity; NS, nodular sclerosis; CR, complete response.

through the protease granzyme B, which, once released from CTLs, is translocated into the target cell by perforin, where it activates the effector caspase cascade [184].

Alterations observed in the G1-S checkpoint of H/RS cell cycle, in the principal tumor suppressor pathways Rb-p16INK4a and p27KIP1, and the high rate of proliferation (MIB1, BCL6) are also strongly associated with higher infiltration of the overall immune response against the tumor [178, 185, 186]. Cytotoxic cells are able to induce directly the permanent downregulation of p27KIP1, probably as a consequence of increased degradation mediated by SKP2, an ubiquitin ligase for p27KIP1 [186–188]. Related with the heightened proliferative state in these tumors is the high level of expression of Bcl6, a multifunctional regulator that is able not only to downregulate cyclin D2 and p27KIP1 expression [189] but also to repress Bcl-XL [190].

The presence of EBV was significantly associated with the overexpression of STAT1 and STAT3. STAT3 was found to be associated with a low infiltration of CD4 T lymphocytes and a high infiltration of activated cytotoxic cells. Although STAT1 is considered to be a potential tumor suppressor (promoting apoptosis), STAT3 is thought to be an oncogene because it leads to the activation of cyclin D1 and Bcl-XL expression and is involved in promoting cell cycle progression and cellular transformation and in preventing apoptosis [191].

6. Impact of Viruses Infection in HL Microenvironment

Early epidemiologic data suggested that HL develops among persons with a delayed exposure to a ubiquitous infectious agent such as EBV [192]. EBV, a γ herpesvirus with a worldwide distribution, is present in H/RS cells of 40%–60% of cHL lesions and contributes to their pathogenesis [193, 194]. EBV$^+$ H/RS cells express LMP1, LMP2A, LMP2B, the EBV nuclear antigens 1 (EBNA1), and the EBER RNAs, but consistently lack EBNA2 (latency II) [195, 196]. LMP1 is likely to contribute to survival and proliferation of H/RS cells through activation of NF-κB and AP-1 [197, 198]. It is also possible that EBNA1 and the EBERs contribute to the rescue of H/RS cells from apoptosis [199, 200].

The intratumoral immunological alterations induced by EBV$^+$ H/RS cells remain unclear. The abnormal network of cytokines/chemokines and/or their receptors in H/RS cells is involved in the attraction of many of the microenvironmental cells into the lymphoma background. There is increasing evidence suggesting a change in the balance between Th1 and Th2 cells in the pathogenesis of HL and that this change induces reactivation of latent viral infections, including EBV. EBV-infected H/RS cells were shown to stimulate also the stromal production of particular chemokines such as the interferon-inducible chemokine IP-10 (CXCL10) [103] Rantes/CCL5 [201, 202], the ligand CCL28 [203], CCL20 that is capable of attracting regulatory T cells [204], and the macrophage-derived chemoattractant (MDC)/CCL22 [205]. The observation of Th1/antiviral response in EBV$^+$ cHL tissues provides a basis for novel treatment strategies [28, 206]. The role of Gal-1, which has been shown to be selectively overexpressed on H/RS cells, was also examined in the context of EBV-specific CD8$^+$ T-cell responses in HL. Its expression was associated with a reduced CD8$^+$ T-cell infiltration and more specifically with an impaired response towards LMP 1 and 2. Moreover, the in vitro exposure to recombinant Gal-1 inhibited proliferation and interferon-gamma expression by EBV-specific T cells [29].

A low proportion of CD4$^+$ cells appears also to be significantly related to EBV status, probably due to the relation with the local tumor-associated suppression of EBV-specific T-cell responses observed in EBV$^+$ HL cases [207]. In the case of immunosuppressed patients, HIV infection affects, for direct or indirect mechanisms, both reactive changes as neoplastic lymphoid tissue. Recently we have seen a significant loss of intratumoral T cells CD4$^+$ (CD4/CD8 ratio reversal) and a decrease in intratumoral activated CTL in patients with HIV-infected HL [208].

A link between septic environment, the high prevalence of Th17 cells, and the favorable outcome impact of intratumoral regulatory T cells (Tregs) has been postulated. The putative role of the dense microbiological flora present in the large intestine with a trend toward translocation through the tumor has been emphasized to explain the favorable outcome of patients bearing colorectal carcinoma (CRC) with a high Tregs infiltration [209]. This microbiological hazard requires a T-cell-mediated inflammatory antimicrobial response that involves Th17 cells. This Th17-cell-dependent proinflammatory and tumor-enhancing response can be attenuated by Tregs, thus constituting a possible explanation for their favorable role in CRC prognosis. In HL, characterization of the inflammatory cytokine profiles in EBV-HL patients revealed elevated Th2 and Th17 responses [210].

7. Therapeutic Strategies to Overcome Immune Escape in HL

In the last years, numerous studies have revealed the critical importance of the microenvironment in the evolution and progression of HL after antineoplastic treatments. This fact has opened new ways for clinical research taking into account the impact of the classical and new immunogenic agents over the characteristic Hodgkin's microenvironment, envisioning alternative treatment strategies (Figure 5).

7.1. Chemotherapy. Chemotherapy remains the therapeutical modality of choice for the systemic treatment of HL for curative purposes. Impact of conventional chemotherapy on the relationship between the tumor and the immune system seems to be crucial. Some groups have confirmed that cell death induced by chemotherapy imply a variety of immune reactions that mediate a sort of vaccination effect via release of an "antigenic milieu" that, in turn, may represent the major determinants of the therapeutical success of this treatment in lymphoproliferative syndromes [211]. Preclinical studies have demonstrated that immune stimulation might be mediated by chemotherapy in murine cancer models treated with gemcitabine and doxorubicin [212, 213]. The explanation to this selective immune activation is an increased CD8 T lymphocyte expansion and an increased density of TIL mediated by an effective MHC class I cross-presentation of tumor antigens released and phagocytosed [214].

Thus, there are now clear evidences supporting the fact that drugs like anthracyclines, cyclophosphamide, or gemcitabine may promote apoptosis in tumor cells with immunogenic effects through several mechanisms [215]. This sort of immunogenic tumor cell death is characterized by a temporal sequence of events including early translocation of calreticulin (CRT) 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 high-mobility group protein B1 (HMGB1). HMGB1 is able to bind to the toll-like receptor 4 (TLR4) on DC, which allows tumor-derived antigens to be processed and presented along with MHC and costimulatory molecules on the surface of DC [216]. These mechanisms altogether serve to trigger DC-mediated specific antitumor response, which may be enhanced by the use of costimulatory molecules [217]. In addition, other more general effects of chemotherapy on the surrounding stroma are postulated like secondary necrosis or eradication of tumor cells [218]. Gemcitabine has demonstrated the ability to restore immune surveillance by reducing MDSC levels in murine models [219].

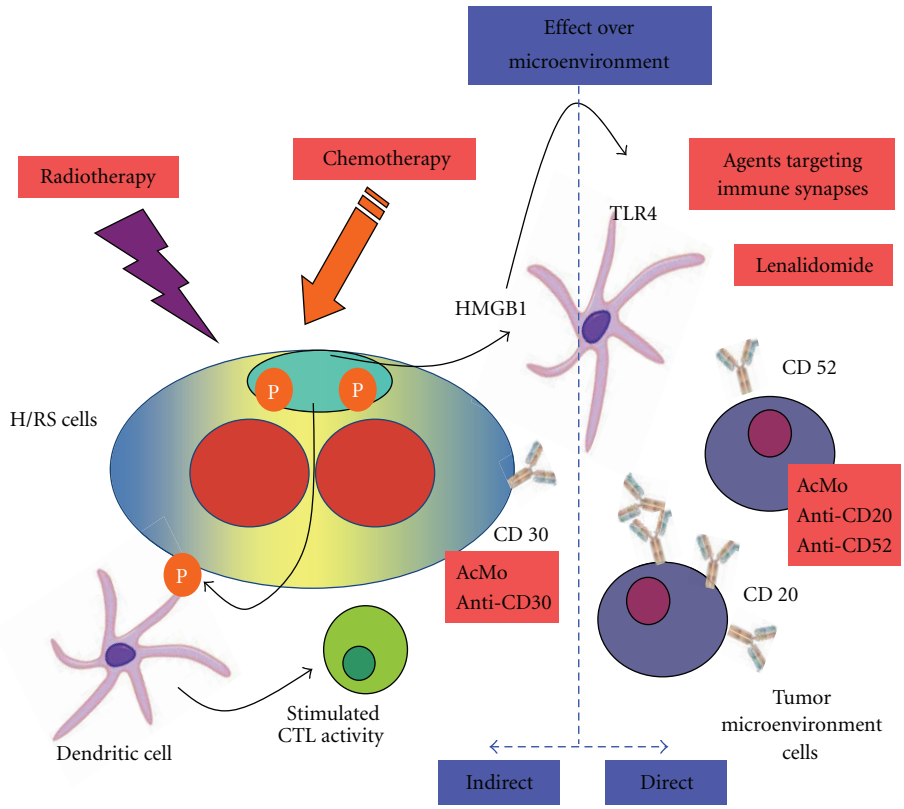


Figure 5: Therapeutic strategies to overcome immune escape in HL. AcMo: monoclonal antibodies. H/RS cells: Hodgkin's/Reed-Sternberg cells. CTL: cytotoxic T lymphocytes. HMGB1: high-mobility group protein B1. TLR4: Toll-like receptor 4.

In conclusion, emerging evidence led to postulate a paradigm shift in the way of understanding the effects of CT on the surrounding stroma [220, 221]. These new findings may serve to consider chemotherapeutics like anthracyclines and gemcitabine as less empirical and more specific drugs, and thus may help to customize treatments in HL taking into account their potential effects on the microenvironment.

7.2. Radiotherapy. Radiation therapy still plays a major role in the management of HL. Ionizing radiation can induce a cascade of pro-immunogenic and proinflammatory effects. Different responses have been described: major histocompatibility complex induction, release of specific antigens, and chemokines production. All these mechanisms are triggered from the radiotherapy target area, neighborhood tissues, and also from the systemic immunological response. These inflammatory effects can convert the irradiated site into an immunogenic hub, by engaging both the innate and adaptive immune response [222, 223]. Locally ionizing radiation promotes the differentiation of monocytes into macrophages with an M1 phenotype (tumor inhibiting) and also the release of radiation-specific antigens that promotes the development of a sustained and effective adaptive immune response to the tumor [223, 224].

The immune-modulating effects of radiation are influenced by several factors. Low radiation dose activates innate immune cells and fails to induce cell death, and thus induces a protumorigenic effect over the immune system [225, 226]. Otherwise, significant radiation dose leads to cell death and induces specific signals that are sensed by innate immune cells, which generate an antitumor immunity.

Classically, apoptosis has been considered as a nonimmunogenic event. Nevertheless, as happens with anthracyclines, radiotherapy might induce apoptosis due to an immunogenic mechanism. Both therapies translocate CRL from the endoplastic reticulum to the cell surface. The superficial expression of CRL in tumor cells causes an important tumor immune response with effective recognition and phagocytosis by DC, leading to cytotoxic T-cell response. In the same manner, a cytotoxic response mediated by $CD8^+$ T cells occurs when heat shock proteins (HSP) such as HSP70 and HSP90 are transferred to the plasma membrane. HSP also stimulate natural killer-mediated cell lysis, through NKG2A ligands. Secondary, DCs mature and release proinflammatory cytokines, such as HMGB1, which binds to toll-like receptor 4 (TLR4). Those mechanisms enable antigen processing and presentation [215, 227].

An accurate radiotherapy treatment has increased its interest under an immune point of view. Effectively, the reduction of naïve T-cells account after an irradiation of drainage lymphs without disease can lead to a distal reactivation of malignancies T cells [222].

The radiation abscopal effect is described as the reduction of the tumor growth outside the treatment field, and some clinical cases of this effect has been reported in

Table 2: Clinical experience with new immunotherapies in Hodgkin lymphoma.

Agent	Mechanism of action	Clinical development status	References
Ipilimumab	Anti-CTL4 Mo Ab	Phase I	[245]
Rituximab	Anti-CD20 Mo Ab	Pilot studies Phase II (combined with CT)	[246–248] [249]
90Y-ibritumomab tiuxetan	Anti-CD20 radio-immunoconjugate	Pilot studies	
Alemtuzumab	Anti-CD52 Mo Ab	Pilot-phase II studies	[250, 251]
Lenalidomide	Immunomodulatory	Phase II	[252–256]
Brentuximab vedotin	Antibody-drug conjugate (anti-CD30 plus tubulin destabilizer)	Phase II	[257, 258]

Mo Ab: monoclonal antibody. CT: chemotherapy.

different tumor types, including lymphoma [228–230]. Their mechanisms and therapeutic approach are not well elucidated [231]. However, main hypotheses imply that local irradiation induced a release of systemic cytokines that mediate an immune antitumor effect and/or the fact that local irradiation might induce systemic tumor specific T-cell responses.

Preliminary results promise that immunotherapy may serve as booster, amplifying immune effectors triggered by radiotherapy as exemplified in experiments that combine it with anti-CTLA-4 monoclonal antibodies or costimulators such as GM-CSF, interferons, or IL-2. Radiotherapy can induce cancer cell death that is mediated by the host's immune system. Elucidation of these mechanisms might offer advantages in the cytotoxic therapy.

7.3. Immune Synapses as Therapeutic Target in HL. The immune synapse is a region of physical contact between the T cell and the antigen presenting cell (APC) and it represents one of the major determinants of the immune response against tumoral antigens [232]. Two main signals are required for an effective T-cell activation. The first signal is provided by the recognition of cognate antigen bound major histocompatibility complex (MHC) by the T-cell receptor (TCR) [233]. Additional costimulatory signals are provided by engagement of coreceptors. The canonical coreceptor CD28 binds to members of the B7 family present on APC. However, soon after T-cell priming, other negative regulatory molecules are induced on T-cells leading to downregulation of the T-cell response [234]. Some of the main molecules that act as immune checkpoints on the immune synapse are CD40 and OX40 with costimulatory properties and CTLA-4 and PD1 that induce coinhibitory effects. Preclinical data support an eventual role of the drugs targeting these molecules in HL.

CTLA-4 acts as a key negative regulator of CD28 dependent T-cell activation [218]. CTLA-4 is produced and mobilized from the internal side of the cell membrane, to the immune synapses 2 to 3 days after T-cell activation has taken place. There, it is bound to either one of the costimulatory molecules, CD80 and CD86. CTLA-4 expression turns the activated T cell to an inhibitory T cell. A delay in CTLA-4 expression favors T-cell activation and could be a pathway to improve or expand the immune response against tumors [218]. There are two CTLA-4-blocking antibodies for use in humans that have been mostly tested in patients with metastatic melanoma [235]. Recently, the fully human immunoglobulin G1 (IgG1) monoclonal antibody ipilimumab (Bristol-Myers Squibb, Princeton, NJ, USA) has demonstrated significant benefits in overall survival in randomized phase III studies in the first- or second-line treatment of metastatic melanoma [236, 237], gaining FDA approval. Clinical research of anti-CTLA-4 in hematologic tumors has been scarce to date. However, a phase 1 dose escalation trial with ipilimumab in the setting of allohematopoietic cell transplantation for relapsed hematologic malignancies reported interesting results [238] (Table 2). This trial tried to assess the efficacy of ipilimumab in augmenting the graft versus malignancy (GVM) effect. Among 14 patients with relapsed HL, 2 achieved a durable complete response and other 2 patients who had evidence of rapid progression achieved disease stabilization after ipilimumab [238]. This clinical effect in a highly pretreated population represents a proof of principle of activity of the anti-CTLA4 antibodies in HL and merits further investigation.

PD-1 is expressed on activated T and B cells, natural killer, dendritic cells, and activated monocytes [239]. PD-1 plays a major role in maintenance of T-cell tolerance limiting effector T-cell responses. There are two ligands of PD1, PD-L1, and PD-L2 (or B7-H1 and B7-H2) [240]. PD-L1 is aberrantly expressed in H/RS cells of Hodgkin's lymphoma and thus it can induce immune suppression through signaling PD-1 [33]. PD-L1-PD-1 signaling system is operative in patients with HL, and TILs around H/RS cells seem to be kept in balance by this inhibitory signaling. These findings suggest a plausible mechanism for deficient cellular immunity observed in HL patients and propose a potentially effective immunologic strategy for the treatment of HL.

CD40 is a member of the tumor necrosis factor receptor family expressed on macrophages, dendritic cells, endothelial and B cells, and fibroblasts [241]. Binding of CD40 with its CD40 ligand (CD40L) or CD154 acts on APC and T cells mediate both cellular and humoral responses. Specifically on APC, CD40 plays a central role in priming and expansion of antigen-specific CD4 T cells by regulating the expression

of costimulatory molecules on APC such as CD80 and CD86 (B7.1 and B7.2) and by production of cytokines such as IL-12, IL-8, or TNF-α [242]. The functional role of CD40/CD40L and interferon regulatory factor 4 (IRF4) in Hodgkin's lymphoma microenvironment seems to be extremely important in HL [243]. A phase I study of the humanized anti-CD40 monoclonal antibody dacetuzumab in 50 patients with refractory or recurrent non-HL has been performed showing an acceptable safety profile and modest activity with 6 objective responses reported [244].

OX-40 is a member of the tumor necrosis factor (TNF) superfamily that needs T-cell activation to be expressed [259]. OX-40 is present in $CD4^+$ and $CD8^+$ T cells, whereas its ligand OX40L is expressed on activated APC, B cells, and macrophages [260]. Engagement of OX40L with the OX40 receptor is essential for the proliferation and survival of T cells leading to a larger expansion of effector T and antigen-specific memory T cells [260]. In addition, OX40 signaling increases cytokine secretion by $CD4^+$T cells and enhances the development of Th1 and Th2 cells. Recently, it has been demonstrated that histone deacetylase inhibitors (HDACIs) may have a favorable antitumor effect by regulating the expression of OX40L in HL [261]. Clinical responses achieved in relapsed and heavily pretreated HL with some HDACIs like vorinostat, mocetinostat, or panobinostat might be mediated by the upregulation of OX40L in HL cells [262].

7.4. Monoclonal Antibodies Targeting HL Microenvironment. CD20 and CD52 molecules are not commonly expressed on the H/RS cells; however, the surrounding cells of the characteristic HL microenvironment commonly express extensively these antigens. Thus, interfering the crosstalk between H/RS cells and their cellular partners with monoclonal antibodies against CD20 and CD52 may represent an attractive therapeutic strategy to explore in clinical research.

The monoclonal antibody anti-CD20 rituximab is one of the therapeutic strategies aimed to deplete the HL microenvironment of normal B cells required for tumor cell growth. Specifically in classical HL, it has shown activity as single agent. In a pilot study, 5 out of 24 heavily pretreated patients with relapsed/refractory cHL treated with rituximab achieved a clinical response [247]. Interestingly, responses were achieved in patients with CD20-H/RS cells. Rituximab has also been tested combined with chemotherapies like ABVD and gemcitabine. Specifically with gemcitabine as salvage therapy it has demonstrated surprising and unexpected high overall response rates (48–88%) [248] (Table 2). Impact of rituximab on tumor microenvironment by depleting benign $CD20^+$ cells is postulated as the main antineoplastic mechanism of action of this drug in HL, independently of CD20 expression on the RS cells. Reactive B-cell depletion in HL is being further tested by the use of anti-CD20 radioimmunoconjugates (90Y-ibritumomab tiuxetan and 131I-tositumomab) with pilot studies completed reporting favourable results in terms of tumour response and symptom control [263].

Alemtuzumab, a humanized IgG1γ monoclonal antibody directed against CD52, has shown notable activity as monotherapy for chronic lymphocytic leukemia [264]. Although its detailed mechanism of action is not completely clear, the binding of alemtuzumab to CD52 on target cells may cause cell death by three different mechanisms: complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC), and apoptosis [250]. Since RS cells of HL do not express CD52, only surrounding cells such as neutrophils, eosinophils, macrophages, mast cells, and B and T cells, that strongly express the CD52 antigen, would be the targets for alemtuzumab and thus might be depleted by this antibody, depriving the RS cells of their critical survival factors. Although clinical trials are lacking in HL, results from a reduced-intensity conditioning allotransplantation study in relapsed HL suggest that alemtuzumab-induced elimination of infiltrating T cells may critically impact on the efficacy of the procedure and on the ability of donor lymphocytes in eradicating residual malignancy [251] (Table 2).

7.5. Lenalidomide. Other biological compounds with significant effects upon tumor microenvironment like lenalidomide are under clinical investigation, and at this moment represent one of the most promising therapeutical strategies in HL [253]. Lenalidomide (Revlimid), a thalidomide-derivate, belongs to a novel class of immunomodulatory drugs (IMIDs) approved for the treatment of multiple myeloma and myelodysplastic syndrome with deletion (-q5) [253]. Lenalidomide has multiple modes of action, including direct induction of apoptosis in tumour cells, antiangiogenic effects, and the activation of immune cells, such as natural killer cells and T cells, enhancing Th1-type cellular immunity and natural killer T-cell cytotoxicity [253]. Preliminary results of some clinical trials of lenalidomide in HL have been recently reported [254–256]. In all studies, administration of oral daily lenalidomide in heavily pretreated HL patients induced clinical response ranging from 17 to 50%, and in most other patients disease stabilization was achieved. This pattern of response appears fully compatible with the predicted actions of lenalidomide towards the HL microenvironment. Results of a serie of HL treated with lenalidomide were of great interest at this point [265]. 12 patients with relapsed or refractory HL were included in this program. All patients had relapsed after at least four chemotherapies, and, except two patients, all had previously undergone high dose chemotherapy and autologous stem cell transplantation. Most patients had not responded to the previous treatment. With respect to clinical outcome, none of the twelve patients showed radiological evidence of progression after two cycles of lenalidomide. Overall response rate was 50% (6 of 12), with 5 partial responses and 1 complete remission, in addition six patients had stable disease after two cycles [265] (Table 2).

7.6. Anti-CD30 Monoclonal Antibodies. The member of the tumor-necrosis-factor- (TNF-) receptor family CD30 is expressed abundantly on Reed-Sternberg cells of HL [266]. CD30 has pleiotropic biologic functions, being capable of promoting cell proliferation and survival as well as inducing

antiproliferative responses and cell death. Final effects of CD30 activation seem largely dependent on the microenvironment context [266]. Unconjugated anti-CD30 antibodies have been tested in phase I and II studies showing limited clinical activity. On the contrary, the use of antibody-drug conjugates (ADCs) has rendered better results [267]. Brentuximab vedotin (SGN-35) is an ADC consisting of chimeric anti-CD30 antibody cAC10 (SGN-30) conjugated to the tubulin destabilizer monomethyl auristatin E (MMAE) [267]. In the first in human phase I dose escalation study, brentuximab vedotin was administered to 45 patients with relapsed or refractory CD30-positive lymphomas, primarily HL and anaplastic large cell lymphoma (ALCL) [257]. Brentuximab vedotin showed a good safety profile and objective response was observed in 17 (38%) patients, including 11 (24%) complete remissions. Tumor regression was observed in 86% of patients. Results of a phase II trial in relapsed HL has been recently communicated [258]. 102 patients were enrolled with a median age of 31 years and all were required to have failed an ASCT. Overall response was achieved in 76 of 102 patients (ORR: 75%) with 35 complete responses (CR: 34%). After these impressive results, brentuximab was recommended for an accelerated approval by the FDA and in August 2011, was approved in the US for the treatment of HL after failure of autologous stem cell transplant (ASCT) or after failure of at least two prior multiagent chemotherapy regimens in ASCT-ineligible candidates (Table 2).

8. Conclusions

The recent research activities led to a better understanding of the phenotype, molecular characteristics, histogenesis, and possible mechanisms of HL lymphomagenesis. New pathologic factors have been studied recently, showing that HL can be differentiated through its specific cellular microenvironment. The interplay between tumoral cells and the reactive microenvironment determines not only the histological morphology and classification but also the clinicopathological features of HL patients. Importantly, this may correlate also with the clinical course of disease and the final long-term outcomes. However, recent advances in our understanding of HL biology and immunology seem to indicate that infiltrated immune cells in the tumoral microenvironment may play different, even opposite, functions according to the signals it senses. It is critical to understand what happens in the tumoral microenvironment in order to design fine tune approaches that may modulate immune response toward cancer cell destruction. Strategies aimed at interfering with the crosstalk between H/RS cells and their cellular partners have been taken into account in the development of new immunotherapys that target different cell components of HL microenvironment. Combination strategies of chemotherapy, especially with anthracyclines and gemcitabine, radiotherapy, and immunotherapy will eventually synergize and obtain meaningful clinical results.

In our opinion there exist large amount of data which provides sufficient evidence to consider the host immune reaction as one of the main determinants of the clinical evolution in HL. Importantly, this immune response is capable of being modulated in clinic, so new therapeutical strategies based on combinatorial approaches with the ability of boosting immune responses might not be neglected in the coming future to improve the chance of cure of our patients with HL.

Authors' Contribution

L. C. Merino, M. Lejeune, and T. Álvaro have contributed equally to the redaction of this paper.

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Review Article

Lessons from Cancer Immunoediting in Cutaneous Melanoma

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We will revisit the dual role of the immune system in controlling and enabling tumor progression, known as *cancer immunoediting*. We will go through the different phases of this phenomenon, exposing the most relevant evidences obtained from experimental models and human clinical data, with special focus on Cutaneous Melanoma, an immunogenic tumor *per excellence*. We will describe the different immunotherapeutic strategies employed and consider current models accounting for tumor heterogeneity. And finally, we will propose a rational discussion of the progress made and the future challenges in the therapeutics of Cutaneous Melanoma, taking into consideration that tumor evolution is the resulting from a continuous feedback between tumor cells and their environment, and that different combinatorial therapeutic approaches can be implemented according to the tumor stage.

1. Introduction

Tumor transformation and progression depends on the cell type and its genetic and epigenetic modifications, where cells overpass several intrinsic tumor suppressor mechanisms and acquire distinctive and complementary capabilities allowing tumor growth and metastatic dissemination [1]. Also, it relies on the interaction of tumor cells with the surrounding environment, the stroma, and the overcoming of extrinsic tumor suppressor mechanisms. In this paper, we will focus on the complex interaction between cancer cells and the immune system, with both controlling and enabling functions, namely, the *cancer immunoediting* theory. In particular, we will discuss the case of Cutaneous Melanoma (CM), a prototypic immunogenic tumor, and include a critical overview of the different immunotherapeutic approaches employed so far.

2. Historical Perspective of the Cancer Immunoediting Theory

The idea that the immune system (IS) is involved in controlling tumor development and progression has been the subject of discussion for many years. In the XX century, Paul Ehrlich stated the theory of cancer immunosurveillance, reformulated in 1957 by Burnet and Thomas, which proposed that the IS is responsible for preventing tumor development in immunocompetent organisms [2]. They reasoned that cancer would be much more frequent in long-lived organisms if it were not for the action of the IS.

The role of IS in tumor control remained controversial until the development of improved genetically-modified murine models of immunodeficiency in the 1990s. Previously, the use of athymic nude mice has mistaken this concept, because no differences were found in tumor incidence between nude and immunocompetent wild type mice [3]. Nowadays, we know that nude mice are not fully immunodeficient, as they have NK cells and some extrathymic T-cell populations [4]. The first supporting evidence proceeded from a landmark work from Robert Schreiber's group, in which the role of IFN-γ in tumor surveillance was proven by demonstrating an increased incidence of chemically induced or spontaneously arising tumors in genetically-modified mice deficient for IFN-γ or all IFN receptors (Rc) (Stat-1-deficient mice), with respect to immunocompetent wild type mice [5]. Similar results were obtained for perforin in a model of spontaneous lymphoma, standing out the relevance of lymphocyte cytotoxicity (NK, NKT, and CD8 cells) in

preventing tumor development [6]. Later on, the role of NK and NKT cells in protection against carcinogenesis was shown in different experimental models [7]. It was getting clear that mice that lacked components of the innate or the adaptive IS would have a dramatically increased rate of tumor formation. In this regard, additional experiments were performed, revealing that immunodeficient mice were more susceptible to carcinogens than immunocompetent mice [8]. Moreover, it was evidenced that the IS not only controls the number of tumor cells but also their immunogenicity, as tumors developed in immunodeficient mice were more immunogenic (unedited) than similar tumors developed in immunocompetent mice (edited). Therefore, the IS would be involved both in tumor development and in tumor edition of immunogenicity [8]. This stands for the theory of *cancer immunoediting*, where the IS has a dual role, both suppressing and enabling cancer. It can repress tumor growth by killing cancer cells or arresting proliferation, but it can also enable tumor growth, either by the selection of less immunogenic cells better adapted to survive in an immunocompetent host, or by the establishment of a tumor-permissive microenvironment that enables tumor growth.

3. The Mains of Cancer Immunoediting

Several experiments were performed in different immunodeficient murine models, where spontaneous as well as carcinogen-induced tumor development were analyzed, along with the study of immunodeficiency's effect on genetically engineered murine tumor models, which all support and contribute to describe the cancer immunoediting process (reviewed in detail in [9]). The cancer immunoediting theory postulates 3 phases that describe tumor evolution in light of its interaction with the IS: elimination, equilibrium, and escape [2]. Cancer cells communicate with stromal cells either by direct contact or by cytokine and chemokine signaling, proceeding in autocrine and paracrine ways to control and shape tumor growth. And it is the integration of all these signals along with the activation state of the different cell types in the tumor environment that determines whether the equilibrium is displaced to an antitumor response, or to a tumor-permissive environment.

3.1. Elimination. This is the immunosurveillance phase, in which both innate and adaptive immunity work together to detect and destroy tumor cells. This process is an extrinsic tumor suppressor mechanism that acts on cancer cells, in which intrinsic tumor suppressor mechanisms have already failed. In the beginning of tumor development, dying tumor cells and damaged-surrounding tissues release factors like IFN-γ, IFN-α/β, and DAMPs [10–12]. These signals recruit cells from innate (NK, NKT, $\gamma\delta$ T cells, macrophages, and dendritic cells) and adaptive IS (CD4 and CD8 T cells). Tumor cells expressing NKG2D activate NK cells. Tumor infiltrating NK cells and macrophages activate each other by production of IFN-γ and IL-12, and kill tumor cells by apoptosis via TRAIL, perforins and reactive oxygen and nitrogen species. The activation of dendritic cells promotes the induction of an adaptive immune response, through tumor antigen (Ag) presentation to CD8 cytotoxic T cells (CTL) with help from CD4 cells, ideally generating a long-lasting immune response. Tumor Ag were first evidenced thanks to the finding that mice immunized with carcinogen-induced tumors were protected in case of a new challenge with the same tumor [13]. There are different types of *tumor Ag*, including those coded by aberrantly expressed normal genes (melanocyte differentiation Ag in CM); tumor-mutated genes (p53); cancer-testis genes, that in physiological conditions are only expressed in germ cells (MAGE and NY-ESO-1); and genes encoding viral proteins (HPV proteins).

If the tumor is completely destroyed by the IS, the elimination phase would complete cancer immunoediting. It is important for early tumor control its origin (spontaneous or induced by a carcinogen) as well as its anatomic localization and growth rate. Nowadays, we know that the IS prevents cancer development by different ways: it protects the host from viral infections; it prevents an inflammatory environment that enables tumorigenesis by abruptly removing pathogens; and it eliminates tumor cells by effector cells from the innate and adaptive IS.

In a recent controversial work where, oppositely to the traditional model of primary tumor progression to metastasis, it was proposed that tumor dissemination to secondary organs would be an early event upon transformation, but cancer cells would remain in a dormant state, resulting in staggered metastatic outgrowth [14]. In a murine model of spontaneous melanoma, tumor cells were found to disseminate early in the development of the primary tumor and remain dormant according to the tissue. Dormant cells from lung showed low proliferation rate in comparison to primary tumors, which was partly mediated by cytostatic $CD8^+$ T cells. Therefore, immune strategies that favor the dormancy of disseminated cells can control the development of metastases.

3.2. Equilibrium. This phase takes place when a group of tumor cells survive the initial attack from the IS and move into an equilibrium phase, in which tumor cells are controlled by the IS but cannot be completely eliminated. In this way, tumors can be controlled by the IS for long periods of time, encompassing the host's entire life. T cells, IL-12, and IFN-γ are known to sustain the dormancy state [15].

Tumor cells may remain quiescent, with no cell division or apoptosis [16]; or may proliferate and become balanced by apoptosis, with no increase in number [17]. Continuous interaction of the tumor with the IS may lead to the edition of tumor immunogenicity, where cancer cells are modified, generating less immunogenic tumor variants that may escape control by the IS, proliferating and developing clinically detectable tumors.

An experimental model of equilibrium was established by administrating low doses of carcinogen MCA (3-methylcholanthrene) in wild type mice, which was interrupted when tumors arose after CD4, CD8, and IFN-γ depletion [15]. However, the same experiment performed in immunodeficient $Rag^{-/-}$ mice did not introduce any change, meaning that disruption of equilibrium would not occur as a result of prolonged *de novo* transformation. Careful examination

of the stable mass at equilibrium revealed the presence of atypical cells, with low proliferation index, that induced tumor formation when transplanted into immunodeficient mice. And edited cells from arising tumors were found to be less immunogenic than unedited cells from equilibrium. These experiments showed that cancer cells in equilibrium proliferate poorly and remain unedited, until they spontaneously become edited and escape immune control and grow.

3.3. Escape. Continuous pressure by the IS on genetically unstable cells can lead to the generation of tumor variants that (i) are no longer recognized by the IS, (ii) become insensitive to effector mechanisms, and (iii) induce an immunosuppressor, tolerant microenvironment. Also, changes in the IS are induced that might contribute to a tumor-permissive environment. As a consequence, tumors progress. In Table 1, several of the mechanisms involved in tumor escape are described, including changes in tumor cells and in IS cells, interfering especially with innate and cellular immune response. Supporting references are provided both by experimental models and clinical data from human patients. We will focus on CM, a prototype immunogenic tumor.

4. Cutaneous Melanoma: A Test Field for Immunotherapy

CM is the neoplasia originated from melanocytes that develops in the skin, and it has the fastest growing incidence worldwide [18]. At the clinical-histological level, the Clark model proposes a gradual transition from normal melanocytes to dysplastic nevi, then to primary CM, including radial and vertical growth phases, eventually leading to metastasis (mts) to the lymph nodes (LN) and distant organs [19]. Early diagnosed tumors (stages I-II, AJCC) are curable by surgery in more than 90% of cases; however, when CM metastasizes, only a minority of patients can be cured [20]. CM would not respond to conventional therapies like radiotherapy and chemotherapy; nevertheless, as it is an immunogenic tumor, it allows the use of immunotherapy as an alternative. Among the strongest supporting evidences for the dual role of the IS in CM eradication and progression are included the following:

(a) Tumor Antigens. The presence of tumor Ag in humans was shown by modern methodologies, involving the use of antibodies and CTL derived from patients as probes, tested on autologous tumor cell libraries. Among CM Ag outstands melanocyte differentiation Ag (MD-Ag) such as MART-1 [21, 22], gp100 [23], tyrosinase [24], tyrosinase-related protein-1 (TRP1) [25], TRP2 [26], and MELOE-1 [27]; cancer-testis Ag from the MAGE super-family [28] and NY-ESO-1 [29]; and tumor-mutated Ag such as BRAF [30].

(b) Spontaneous regressions. The finding of both humoral and cellular immunity to tumor Ag suggests that the IS is capable of eliciting a coordinated immune response to tumors as it would to a foreign Ag. Indeed, several tumor regressions were observed in patients as a consequence of the action of the IS [31]. Infiltration of IS cells is an early event in transformation and it is associated with disease outcome. There are several studies in CM patients that correlate quantity, quality, and distribution of tumor infiltrating lymphocytes (TIL) with patient survival [19, 32, 33]. The first studies just analyzed the presence and distribution of lymphocytes; more recent studies also focus on the immunophenotype of IS cells, as it is known that the IS may move from an antitumor environment to a tumor-permissive one. It was described in a case report a CM patient treated with anti-CTLA-4 therapy that was undergoing simultaneously the three phases of cancer immunoediting, with regressing, stable, and progressing lesions [34]. Probably, the environment of the different metastases (mts) would account for tumor response. In another clinical case, different rounds of immunoediting, escape and immune adaptation by shifting of the T-cell response were observed [35].

(c) Immunodeficiency. In general, immunodeficiency is associated with an increased risk of developing cancer. Most related factors include viral oncogenesis and reduced tumor immunosurveillance. Immunocompromised patients, like transplant recipients or AIDS patients, develop lymphomas (Epstein-Barr virus), Kaposi's sarcoma (Herpes virus), and cervical cancer (Human papillomavirus) [36]. An increased incidence of tumors non-related to virus, like colon, lung, pancreas, kidney, head and neck, skin carcinomas and CM was also observed [37]. In a case report, it was described that two patients that received kidney grafts from a common donor developed CM. It was further revealed that the donor had overcome this pathology in the past; therefore, the donor's kidney probably contained CM cells held in equilibrium by the IS. When kidneys were grafted into immunosuppressed recipients, the development of CM was favored [38]. These evidences are consistent with the idea that tumors progress in immunosuppressive permissive environments.

(d) Immunosuppression. Although CM is highly immunogenic, tumors develop and progress in immunocompetent patients. One of the contributing factors is the induction of a local state of immune suppression and tolerance to tumors as a result of tumor interaction with its environment. Cancer cells develop different mechanisms for tumor escape, including evasion of Ag recognition by the IS and secretion of immunosuppressor and proapoptotic factors (Table 1). Analysis of immunosuppressor factors in primary CM biopsies, negative and positive sentinel lymph nodes (SLN), and LN with advanced metastasis revealed that primary CM cells secreted TGF-β2 that renders dendritic cells tolerogenic; tolerogenic dendritic cells (tDC) and Treg were found at all stages, with increasing IDO and IL-10 secretion with CM progression, making the SLN an immunoprivileged site suitable for metastasis [39]. Thus, tumor cells would secrete immunosuppressor factors that would render IS effector cells into a tolerant phenotype, which in turn would secrete more

Table 1: Mechanisms involved in tumor escape in Cutaneous Melanoma. Examples of changes in tumor and immune cells derived from experimental models (syngeneic and xenograft murine models, as well as *in vitro* human models); and from clinical data from patients are described.

	Mechanism	Description	Examples	
			Experimental models	Clinical data
Changes in tumor cells	Evasion of Ag recognition by the IS	Loss of Ag expression; HLA-I loss; Ag plasticity; fails in Ag presentation	MART-1 loss after specific CTL treatment in xenografts [102]; TYR and TRP-2 loss in CM B16 [103]; HLA-I loss in xenografts [104, 105]; plasticity in MD-Ag and CD271 expression [101]	Tumor immunoediting in subsequent metastases [106]; MART-1 loss [107]; TAP-1 and MART-1 loss [71] β-2 microglobulin loss [108]; HLA-I, MART-1, and TYR loss [109]; gp100, TRP-2, SOX10, NY-ESO-1, and HLA-I loss [110]; MART-1/HLA-I loss after specific ACT [86]; plasticity in several Ag, including ABCB5 and CD271 [95]; plasticity in MD-Ag and CD271 expression [101]
	Secretion of immunosuppressor factors	Factors interfering with NK, macrophages, DC, CD4 and CD8, function	HLA-G secretion in exosomes [111], NKG2DL downregulation [112], and HLA-E expression [113] impair NK/CD8 cytotoxicity. CCL21 expression induces lymphoid-like stroma, and recruits/activates Treg and MDSC in syngeneic and xenografts models [40]	IDO expression recruits Treg enabling mts [114, 115]; ICOS-L promotes activation and expansion of Treg [116]; IL-10 expression recruits Treg & TAM and is associated with CM progression [117]; TGF-β1 expression recruits tDC & Treg [39]; IDO, TGF-β, and CCL17/CCL22 expression in genome-wide association studies [118];
	Secretion of proapoptotic factors	Factors inducing apoptosis in effector T cells (*tumor counterattack*)	FasL expression [119], PD1L (B7-H1) expression [120], and FASL and TRAIL expression induces T-cell apoptosis [121, 122]	Gal-3 expression induces apoptosis in TIL [51]; Gal-3 is a marker of progression [123]; Gal-1 & Gal-3 expression in genome-wide association studies [118]
Changes in IS cells	Natural killer cells (NK cells)	Impaired effector function	Gal-3 impairs NK cytotoxicity in CM B16 [52]	Impaired lytic granule polarization by HLA-G [113, 124]; loss of activating Rc CD161 & NKG2DR expression in CM mts [125]; loss of activating Rc (NKG2DR), increase of inhibitory Rc (CD158b), and impaired activity (lower CD107a, IFN-γ and TNF-α expression) in CM mts [47, 48]

Table 1: Continued.

	Mechanism	Description	Examples	
			Experimental models	Clinical data
Changes in IS cells	Cytotoxic T cells (CTL)/CD8 cells	Impaired effector function	TCR zeta-chain downregulation [126]	Decreased expression of IL-2, IL-4 and IFN-γ by TIL [127]; tolerance induction in mts, with loss of IFN-γ and perforin expression [49]; low proportion of $CD8^+CD27^-$ cytolytic cells in primary CM [50]
	Regulatory T cells (Treg)	They secrete immunosuppressive factors interfering with NK, macrophages, DC, CD4, and CD8 function	Treg inhibit FasL-induced innate and adaptive tumor immunity in CM B16 [128]	Induce immunotolerance in CM genesis [41]; upregulated in LN^+ [129] and advanced CM [130]; Treg impairs NY-ESO-1 vaccine [131, 132]; secrete IL-10 [39]; Treg IDO^+ as a negative survival prognostic marker for SLN^- patients [42]
	Tolerogenic dendritic cells (tDC)	tDCs have diminished Ag presentation and T-cell activation; induce anergy and tolerance in T-cells	P38 MAPK expression drives tDC in CM progression [133]	Upregulation in advanced patients [130]; TGF-RβI promotes tDC, expressing IDO and TGF-β2 [39]
	Plasmacytoid dendritic cells (pDC)	Induce strong immunosuppression	IDO expression and strong immunosuppression in mouse tumor-draining LN [134]	Related to poor prognosis in primary CM [44]; CCR6/CCL20 interaction involved in pDC recruitment to the tumor [135]; pDC associates with SLN^+ [45] and express IDO [46]
	Tumor-associated macrophages (TAM)	M2-polarized TAM release immunosuppressor, proangiogenic, and growth factors	MCP-1 recruits TAM [57]; Adrenomedullin expression by TAM [58] is related to CM angiogenesis and growth.	Cyclooxygenase-2 expression in TAM as a marker of CM progression [59]; TAM impairs NY-ESO-1 vaccine [131]
	Myeloid-derived suppressor cells (MDSC)	Progenitor and immature myeloid cells induce Treg; aminoacids depletion; TCR modification	Treg recruits MDSC and induces B7-H1 and IL-10 expression in CM [60]; chronic inflammation recruits MDSC to tumor and impairs T-cell function [61]; MDSC enables CM mts [62]	Upregulated in advanced CM, MDSC immunosuppress CD4 and CD8 function [63]
	Tumor-associated neutrophils (TAN)	Promote chronic inflammation and tumor migration	Mac-1 (TAN)/ICAM-1 interaction promotes CM cells migration [64]	TAN associated with poor prognosis in primary CM [44], and an independent negative OS factor in advanced CM patients [65]
	Mast cells	Promote chronic inflammatory environment and angiogenesis	Il-8 inflammatory cytokine secretion by CM cells induced by mast cells [66]	Mast cells ($tryptase^+$) association with VEGF expression and angiogenesis in CM [67]; mast cells correlation with poor CM prognosis [68]

immunosuppressor factors preparing the niche for metastasis before tumor dissemination. Recently, it was shown that secretion of CCL21 by melanoma cells promotes tolerance in syngeneic and xenograft CM models [40]. $CCL21^{low}$ tumors presented specific CTL for CM Ag and cytokines related to an immunogenic response. Instead, $CCL21^{high}$ tumors secrete TGF-β1, promote CCR7-dependent Treg and MDSC activation, and increased lymphoid tissue inducer cells, which promoted lymphoid neogenesis.

Regulatory T cells (Treg) are key immunosuppressor factors. The presence of $CD4^+CD25^+FOXP3^+$ Treg was analyzed among different nevi (common/atypical junctional and compound nevi, Spitz nevi) and primary CM [41]. These regulatory cells were found in all these lesions, but were more represented in atypical junctional/compound nevi and in radial growth phase CM, suggesting that Treg induce immunotolerance early during CM genesis, favoring CM growth. Indeed, Indoleamine 2,3-dioxygenase (IDO) expression in Treg, an enzyme with immunosuppressive properties, was identified as a negative survival prognostic marker in SLN^- patients [42]. Moreover, tumor Ag-specific $CD4^+CD25^+FoxP3^+$ Treg were evidenced in the blood of patients with metastatic CM [43]. These cells recognized a broad range of tumor Ag, including gp100, NY-ESO-1, TRP1 and inhibitor of apoptosis protein (IAP), and proliferated in response to specific peptides. They produced preferentially IL-10 and suppressed autologous $CD4^+CD25^-$ T-cell responses in a cell contact-dependent manner; they were not detected in healthy individuals. Therefore, these tumor-Ag-specific Treg might represent a target for improving CM immunotherapy.

Plasmacytoid dendritic cells (pDC) are characterized by the induction of strong immunosuppression. Both pDC and neutrophils were found associated with pSTAT-3 expression in CM, resulting in markers of poor prognosis in primary CM [44]. Also, pDC accumulate in SLN^+ and express IDO, promoting immunotolerance [45, 46].

With regard to *effector cells*, a natural function of NK lymphocytes is to kill cells that fail to express MHC I molecules, thereby contributing to tumor eradication. The most frequent event observed in NK cells during CM progression is loss of activating Rc and increase of inhibitory Rc [47, 48]. With respect to CTL, the induction of an immunotolerant state interferes with the cytotoxic function of CTLs, as IFN-γ and perforin expression decrease [49, 50]. Also, tumor cells secrete factors, like Galectin-3, that induce apoptosis of CTL and NK cells [51, 52]. With regard to tolerance, the functional state of tumor-specific CTLs anti-MART-1 from peripheral blood and metastasis populations from CM patients was compared [49]. TILs expressed lower levels of IFN-γ and perforin than peripheral T cells, indicating a local state of tolerance. However, cytotoxic activity could be recovered after re-stimulation of CD8 cells by *in vitro* culture; therefore, local induction of tolerance would be reversible.

(e) Inflammation. Chronic inflammation is a key factor involved in tumor development and progression (reviewed in [53]). Sun exposure promotes an inflammatory environment in the skin, increasing the risk of developing skin cancer, including CM [54]. Inflammation contributes to tumor initiation by increasing the DNA mutation rate, and through production of reactive oxygen and nitrogen species that induce DNA damage and instability. Also, it activates *tissue repair responses*, inducing proliferation of premalignant cells and enhancing their survival. Tumor-infiltrating IS cells secrete cytokines that activate *key transcription factors* in transformed cells, like NFκB or STAT-3, that control survival, proliferation, growth, angiogenesis, and invasion [55]. In turn, these transcription factors induce chemokines that attract additional inflammatory IS cells to sustain tumor-associated inflammation. Upon transformation, inflammation stimulates angiogenesis and causes local immunosuppression, helping tumor cells to survive and accumulate additional mutations as well as epigenetic changes, enabling tumor progression.

Macrophages are key mediators of the inflammatory response. Macrophages can be classified into M1 and M2 types [56]. *M1 macrophages* are associated with the acute inflammatory response, capable of killing pathogens, and priming antitumor immune responses. They can be activated by IFN-γ and pathogens and express high levels of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-12, or IL-23), MHC molecules and NO (nitric oxide) synthase. On the other hand, *M2 macrophages* (or "alternatively" activated macrophages), induced *in vitro* by IL-4, IL-10, and IL-13, downregulate MHCII and IL-12 expression and increase IL-10, scavenger receptor A, and arginase. This phenotype is related to an inflammatory tumor-permissive environment. However, M1 and M2 macrophages phenotype is plastic since it is defined by gene expression profiles; oppositely to CD4 T_H1 and T_H2 cells, which involved differentiation committed pathways. In CM, M2 *tumor-associated macrophages* (TAM) release tumor-enabling factors, including angiogenic and growth factors. Overexpression of monocyte chemoattractant protein (MCP-1) on CM cells attracted macrophages, enabling tumor growth and angiogenesis in a human xenograft model [57]. Also, adrenomedullin expression by TAM enables tumor growth and angiogenesis in CM B16 [58]. Analysis of TAM at different stages including benign nevi revealed more frequency of $COX\text{-}2^+$ TAM in primary CM, proposing COX-2 as a marker of CM progression [59].

Myeloid-derived suppressor cells (MDSC), a heterogeneous group of progenitor and immature myeloid cells, have emerged as key immune modulators in various human malignancies. In several experimental models, it was shown that chronic inflammation recruits MDSC to the tumor, expressing immunosuppressor factors, impairing T-cell function and enabling metastases [60–62]. MDSC frequency is increased with CM progression, and STAT-3 is a key factor in MDSC development and function [63].

Tumor-associated neutrophils (TAN) were shown to promote CM cells migration via MAC-1/ICAM-1 interaction [64], and to be associated with poor prognosis at all stages [44, 65]. Finally, *mast cells* are also implied in tumor-associated inflammation. TNF-α and histamine secreted by mast cells induced expression of the inflammatory cytokine

IL-8 by CM cells [66]. Tryptase$^+$ mast cells were found related to VEGF expression, enabling angiogenesis in CM [67]; therefore, they were associated with poor prognosis for CM [68].

On this basis, different therapeutic approaches were employed with the main purposes of overcoming chronic inflammation, immunosuppression, and tolerance induced by the own tumor and its environment, and to stimulate tumor Ag immunogenicity and effector function of immune cells in order to eradicate tumors. We will discuss different examples, highlighting evidences from cancer immunoediting, and progresses and challenges in the treatment of CM.

4.1. Adjuvant Therapies. Among drugs used to stimulate immune effector cells, high-dose Interferon alfa2B (IFN-α2b) is a FDA-approved drug for use in patients with stages II-III CM. Whereas it increases disease-free survival and has a moderate effect on overall survival (OS), it is nevertheless related to severe side effects [69]. Another adjuvant therapy commonly employed, interleukin-2 (IL-2), promotes proliferation of T, B, and NK cells. This drug is approved for stage IV CM patients, with 16% objective response (OR) rate, including 6% complete response rate, although associated with short-term acute toxicity [70]. In a case report, a patient presented loss of the TAP-1 and MD-Ag MART-1 within subsequent metastases developed after several therapies, including IL-2 [71]. Cytogenetic analysis of the subsequent metastases revealed similar profiles, indicating a common genetic composition. Sensitivity to previous CTL clones could be restored by introducing MART-1 and TAP-1 by retroviral expression, further supporting the immunoediting of this tumor during its progression.

4.2. Molecular Target-Specific Therapies. Among target-specific therapies are the blockade of oncogenes. The most frequent mutation found in CM (50–65%) is a driver mutation in the BRAF oncogene, BRAF^{V600E}, involved in the MAPK proliferation pathway [30]. Specific inhibitors were designed for BRAF^{V600E} and tested on advanced CM patients. In a phase III study, comparison of the BRAF^{V600E} inhibitor Vemurafenib with the chemotherapeutic drug Dacarbazine showed more than 50% of response rate to Vemurafenib, with a sensitive increase in overall survival and progression-free survival (PFS) in comparison to Dacarbazine [72]. This inhibitor allowed achieving important remissions, although transitory, because relapses were observed. However, the administration of Vemurafenib does not interfere neither with the viability nor functionality of T cells, allowing the implementation of a combinatorial approach with immunotherapy [73]. The increase in the flow of CD4 and CD8 cells to the tumor site after beginning of Vemurafenib administration further supports combinatorial strategies [74]. In a recent landmark work, in MYC$^+$ and BCR-ABL$^+$ lymphoma and leukemia mouse models of oncogene addiction, it was shown that CD4$^+$ cells are involved in cellular senescence, shutdown of angiogenesis and chemokine expression [75]. This provides evidence that the IS plays a role in tumor regression upon oncogene inactivation, a process that was considered cell-autonomous, adding scientific rationale for combination therapeutic approaches.

4.3. Immune Tumor-Specific Therapies. The main routes employed to promote tumor-specific immunity include the active way through the use of *therapeutic vaccines* (*in vivo*), and the passive way through Adoptive Cell Therapy (ACT) (*ex vivo*). Therapeutic vaccines are administered after surgery with the purpose of mounting a long-lasting immunity and controlling any micrometastatic foci. The rational base of vaccines is that tumor Ag must be captured by dendritic cells, which migrate to lymph nodes to activate CD4 and CD8 cells, triggering an adaptive immune response. Therapeutic vaccines include tumor Ag vaccines, with different sources of Ag, like peptides, tumor lysates, recombinant virus, or whole irradiated cells; dendritic cells vaccines, consisting of autologous dendritic cells stimulated *in vitro* with a proper Ag source, maturated *ex vivo*, and then injected back into the patient. ACT involves the expansion of autologous T cells *ex vivo* to achieve sufficient number to eliminate important tumor masses. Either TILs or genetically modified T cells (with clonotypic TCR or chimeric antigenic receptor) might be perfused. This is an attractive approach for advanced patients whose tumors cannot be removed by surgery. However, the convenience of this expensive treatment has not yet been validated in randomized, prospective clinical trials.

With regard to tumor Ag vaccines, in a trial with MART-1, gp100 and tyrosinase peptides in metastatic CM patients, one of the patients who experienced a dramatic tumor regression had preexisting immunity to TRP2 and NY-ESO-1 Ag [76]. Of interest, the immune reactivity against TRP2 persisted over time, whereas that against NY-ESO-1 waned over the course of follow up; it is possible that residual tumors were immunoselected *in vivo* for loss of NY-ESO-1 over time. Other clinical cases of tumor regression upon vaccination with MAGE-1 peptide indicated a higher frequency of CTL towards general tumor Ag (antitumor CTL) than specific vaccine-Ag (anti-vaccine CTL), both in the tumor and in circulation [77, 78]. Antitumor T cells were already present in the patient before vaccination, with some highly dominant clonotypes. Thus, preexisting antitumor T cells may be ineffective at rejecting the tumor either because their frequency is too low, because tumor cells were selected to escape recognition, or because such lymphocytes are functionally deficient. However, this state of functional tolerance might be reversed by the administration of vaccines. A possible explanation is that vaccination induces cytokine cascades both locally and systemically, resulting in activation and proliferation of anti-melanoma Ag precursors, and infiltration of these effectors into tumors. Thus, a spontaneous antitumor T-cell response, which has become ineffective, can be reversed by vaccination and contribute to tumor rejection.

Actually, vaccination with tumor-Ag vaccines has been extensively assayed in CM patients, so far with little success [79, 80]. What could be the reasons for the failure of therapeutic vaccination in a large majority of the patients? A possible explanation is the low occurrence of anti-vaccine T cells that have the required functional properties to migrate to the tumor, resist the inhibitory tumor environment, and

initiate focal activation. Another factor might be the inability to overcome the severe immunosuppressive environment, preventing the effectiveness of any vaccine. In fact, recent studies indicate that selective Treg depletion improves therapeutic effect of vaccines [81]. Another issue is that vaccines that target a unique Ag are in disadvantage, since tumors in general are heterogeneous, CM being not an exception. Therefore resistance may come from the coexistence of heterogeneous populations, or acquired by loss of Ag or HLA expression. In this regard, targeting multiple targets provides a step forward. In this way, previous clinical trials conducted by us indicated that vaccination with allogeneic irradiated cells, in patients in early stages of the disease, may prolong significantly disease-free survival [82]. Also, promising results were obtained for patients with stages II/III CM in a phase I study with autologous dendritic cells loaded *ex vivo* with allogeneic irradiated cells [83, 84].

With regard to ACT, there are also different strategies. Culture of TIL is not suitable for all patients because of technical issues (reviewed in [85]), but achieved 50% OR in different trials [86, 87]. This procedure requires a previous cycle of immunosuppression in order to suppress the endogenous immunosuppressive environment from patients. This OR increased to 72% with more severe immunosuppressive before treatment, but required hematopoietic stem cells transfusion afterwards [88]. Young TIL protocols introduced shorter culture times, although administered TILs were unselected; however, it achieved 50% OR in a phase II study [89]. A limitation of this approach is the requirement that patients have preexisting tumor reactive cells that can be expanded *ex vivo*. Genetically modified T cells are derived from patient's blood cells, therefore are more feasible to be obtained. Cells transduced with most frequent tumor-regression Ag, MART-1 and gp100, allowed to achieve for MART-1 12% OR [90]; and 30% and 19% OR for MART-1 and gp100-specific CTL in another phase I study, including remissions of brain metastases [91]. Studies with cells modified with chimeric antigenic receptors for CM therapy are on the way. The principal disadvantage of specific CTLs/genetically modified T cells is that they target a unique Ag, easing the development of resistance. In a phase I study of TIL immunotherapy, although half of the patients presented clinical responses, almost 60% showed evidence of immunoediting with loss of MART-1 or HLA-I [86]. In other phase-I studies, the effect of MART-1 specific CTL clones in advanced-stage patients was analyzed, with half of the responders showing loss of Ag expression [92, 93].

5. Cutaneous Melanoma Heterogeneity and Immune Response

One question that arises from the observation of limited clinical responses and remissions in Ag-targeted therapies is about the nature of tumor growth and heterogeneity observed in CM. Whether there are different proliferative populations hierarchically organized, with distinguishing Ag, or there are unstable populations with variable proliferative potential. The *cancer stem cell* (CSC) model proposes a cellular hierarchy within tumors in which, as in physiological tissues, only the minor CSC subset would have unlimited proliferative potential, being capable of self-renewal and generation of differentiated cells, accounting for the tumor mass [94]. Oppositely, the stochastic or clonal evolution model states that most cells would self-renew, accounting for tumor growth. A more dynamic model of *phenotypic plasticity* is gaining momentum, in which cells would have a proliferative potential variable in time [95]. This is an important issue, since recent publications described that CM CSC, selected by CD271 expression, would not express MD-Ag MART-1, gp100, and tyrosinase [96]. Also, CM CSC selected by ABCB5, would not express MART-1 [97]. In contrast, it was reported that one out of four CM cells developed tumors in NOD/SCID Il2rg$^{-/-}$ mice without any previous selection [98]; and that phenotypic plasticity, even in CSC markers like CD271 or ABCB5, would be a source of heterogeneity in CM [95].

We were interested in the study of the expression of immunotherapy-relevant Ag in CM proliferative populations. In particular, we wanted to disclose if cells expressing MD-Ag have limited proliferative potential, thus allowing MD-Ag non-expressing clonogenic cells (CC) to survive immune effectors and repopulate the tumor; we also wanted to address if CC would be intrinsically resistant to CTL. We focused on MART-1 and gp100, since in HLA-A0201 patients (40% Latin-Americans), most TILs are directed against them, thus appearing to be the most frequent Ag involved in tumor regressions [99, 100]. We analyzed MART-1/gp100 and the proliferation marker Ki-67 expression in primary and metastatic CM biopsies, observing the coexistence of MART-1/gp100 expressing and non-expressing populations that proliferated competitively, with no differences between primary and metastatic tumors. However, cells with differential proliferative potential might replicate. Therefore, we analyzed MART-1, gp100, tyrosinase, and CD271 expression in colonies obtained from anchorage-independent growing CC of human CM cell lines. By 7 days, colonies displayed positive, negative, and mixed expression patterns. By 14 days, Ag were downregulated, suggesting Ag plasticity. We found that plasticity in MART-1 expression involves promoter methylation. We studied MART-1 and gp100 expression along time in CM growing clones, revealing that Ag levels varied with time without interfering with clonogenicity. Finally, CC MART-1/gp100 expressing cells were efficiently lysed by specific CTL. In conclusion, we found that MD-Ag or CSC marker CD271 expression would not interfere neither with proliferation nor clonogenicity, and CC expressing the proper Ag and HLA-class-I haplotype would not be intrinsically resistant to lysis by CTL. Since MD-Ag-expressing and non-expressing cells are proliferative and clonogenic, giving rise to colonies of thousand cells, both subpopulations should be considered as targets to eradicate tumors [101].

6. Conclusions

What do we know about cancer immunoediting? Extensive research in this field reveals that there is a continuous feedback between the tumor and its microenvironment that

determines tumor fate. It is not a fixed interaction, but rather a dynamic one, where signals from cancer and surrounding cells are constantly modifying each other giving an integral response. However, tumor evolution is progressive, and its study provides the possibility to interfere with this process with different therapeutic approaches according to the tumor stage (*different stages; different approaches*).

What have we learned about CM and immunotherapy? CM is a prototypic immunogenic tumor, with spontaneous regressions described in patients and with several Ag identified. Due to its limited therapeutic options when it metastasizes, immunotherapy has emerged as a remarkable one. In clinical practice, Ag-targeted therapies, even with vaccines or CTL, have achieved modest success. Among contributing factors, the specific blockade of the tumor immunosuppressor environment is a high wall to climb but it is indeed necessary, preserving as much as possible the immune repertoire from the patient. Also, it is relevant to overcome chronic inflammation, which fosters genomic instability, immunosuppression, growth, and angiogenesis in tumors. With regard to tumor cell heterogeneity, it is important to discern whether Ag heterogeneity is due to the presence of differentiated cells with limited proliferative potential (CSC model), or to Ag plasticity independent from proliferation (phenotypic plasticity model), accounting for resistance and escape from immune effectors. We and others found Ag and phenotypic plasticity even in CSC markers. Thus, if Ag expression varies in time, immunotherapeutic approaches should point towards plasticity or multitargeting of Ag, so at least some responder T-cell clones with proper migration capability and resistance to inhibitory factors would be obtained. In advanced patients, the equilibrium phase has largely been displaced, with a highly tumor immunosuppressive environment, many times with unresectable tumors. However, exciting approaches have arisen from studies of CM biology, like the use of $BRAF^{V600E}$ inhibitors; recently, the role of the IS upon oncogene inactivation was evidenced, providing support for combinatorial therapeutic strategies.

What do we learn about cancer immunoediting for improving therapeutic strategies? We learn that the cancer immunoediting process considers the tumor as an integral organ with different components, including cancer cells as well as stromal cells, and so should therapeutic approaches do. Certainly, the continuous study of CM biology and its environment will improve combinatorial therapeutic approaches to reach an equilibrium state or, best of all, achieve tumor eradication.

Abbreviations

ACT: Adoptive cell therapy
Ag: Antigen/s
CSC: Cancer stem cells
CC: Clonogenic cells
CM: Cutaneous melanoma
IDO: Indoleamine 2,3-dioxygenase
IS: Immune system
LN: Lymph node
mts: Metastasis/metastases
MDSC: Myeloid-derived suppressor cells
pDC: Plasmacytoid dendritic cells
PFS: Progression-free survival
OR: Objective response
OS: Overall survival
SLN: Sentinel lymph node
Rc: Receptor
TAM: Tumor-associated macrophages
TAN: Tumor-associated neutrophils
tDC: Tolerogenic dendritic cells
TIL: Tumor-infiltrating lymphocytes.

Conflict of Interests

The authors indicate that they have no potential conflict of interests.

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Review Article

Hematological Malignancies Escape from NK Cell Innate Immune Surveillance: Mechanisms and Therapeutic Implications

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Hematological malignancies treatment improved over the last years resulting in increased achievement of complete or partial remission, but unfortunately high relapse rates are still observed. Therefore, sustainment of long-term remission is crucial. Immune system has a key role in tumor surveillance. Natural killer (NK) cells, at the frontier of innate and adaptive immune system, have a central role in tumor cells surveillance as demonstrated in the setting of allogenic stem cell transplantation. Nevertheless, tumor cells develop various mechanisms to escape from NK cells innate immune pressure. Abnormal NK cytolytic functions have been described in nearly all hematological malignancies. We present here various mechanisms involved in the escape of hematological malignancies from NK cells surveillance: NK cells quantitative deficiency and NK cell qualitative deficiency by increased inhibition signaling or decreased activating stimuli. A challenge of immunotherapy is to restore an efficient antitumor response. A combination of classical therapy plus immune modulation strategies will soon become a standard of care for hematological malignancies.

1. Introduction

Hematological malignancies are cancers that affect blood, bone marrow, and lymph nodes, thus maintaining a slight contact with immune system cells. Although complete remission (CR) rates have increased over the last years, the high incidence of relapse impairs long-term prognosis. While achievement of CR mainly relies on high-dose chemotherapy, long-term control involves maintenance protocols with daily, weekly, or monthly low dose of chemotherapy prolonged for some months or years, as well as the induction or restoration of immune pressure against minimal residual disease (MRD).

Natural killer (NK) cells play a key role in the immune antitumor response as demonstrated by the low relapse rate obtained in allogenic stem cell transplantation when there is a mismatch of NK inhibitory receptors between the host and the graft [1]. NK cells are lymphocytes that have cytotoxic properties and cytokine-production capacities. They are innate immune-effector cells that recognize and kill transformed cells (i.e., tumor cells and virus-infected cells). NK cells also have immune regulatory functions thanks to cytokine and chemokine secretion, which favor the development of a T-helper cell (TH1) response [2].

The role of NK cells in immune monitoring of tumors is essential mainly due to their nonhuman leukocyte antigen (HLA) restricted effect, as the absence or abnormal expression of HLA molecules induces NK-cell cytotoxicity (the so-called "missing self" hypothesis of Karre [3]). Indeed, as a consequence of antigen-specific immune pressure by T-cells, tumor cells with downregulated HLA class I molecules are progressively selected and thus could become targets for NK cytotoxicity.

Phenotypically, NK cells are CD3—lymphocytes that express CD56 and/or CD16 at different levels. CD16 is the FcγRIIIa receptor that can mediate antibody-dependent cell-mediated cytotoxicity (ADCC). When a NK cell has recognized its target cell, it kills the target cell by secreting

cytotoxic granules (perforin, granzyme, and granulysin), *via* interaction with the tumor-necrosis factor family (Fas/FasL) or even by cytokine secretion.

NK cells express a large panel of cell-surface activating and inhibitory receptors that recognize ligands on potential target cells. The balance between the signals delivered by activating and inhibitory receptors determines whether or not NK cells kill a target cell and secrete cytokines [4, 5]. Thus, a "dynamic equilibrium concept" between these receptors controls NK cell activation.

Activating receptors are mainly represented by the natural cytotoxicity receptors (NCR) NKp30/NCR3, NKp44/NCR2, NKp46/NCR1, and NKG2D, but also by coreceptors such as 2B4/CD244 or NTBA. Activating receptors have various ligands (see review [6]), such as the stress-induced molecules HLA class-I chain-related A (MICA) and MICB, which activates cytotoxicity in NK cells through their ligation to NKG2D, leading to the destruction of the target cell. Conversely, NK cell cytotoxicity is downregulated by the engagement of HLA-specific inhibitory receptors (killer immunoglobulin-like receptors (KIR) and CD94/NKG2A/B heterodimers), thus protecting normal cells. The recognition of normal HLA class I molecules on target cells downregulates NK cytotoxicity.

Defects in NK-cell cytotoxicity have been observed in all hematological malignancies [7–10]. The escape of hematological malignancies from NK cell immunity can be explained by general mechanisms that are common to all immune-effector cells (see review [4]), that is, saturation of the immune system by rapid growth of the tumor, inaccessibility of the tumor because of deficient vascularization, but also by dysfunction of the immune system that could be restored by immune modulatory intervention. In this paper, we focus on tumor escape from NK cells surveillance by evocating successively various mechanisms involved (summarized in Table 1): the quantitative deficiency of NK cells, their qualitative impairments caused by increased inhibition, or decreased activation signaling. We then examine the key role of cytokine environments in tumor immune escape from NK cells. Finally, we briefly evocate various therapeutic means to enhance NK cell control of hematological malignancies.

2. Tumor Escape from NK Cell Surveillance: Role of NK Cells Effectors' Quantitative Deficiency

Quantitative deficiency is the first mechanism in myelodysplastic syndrome (MDS) to explain how tumors escape from innate immune surveillance. MDS is a preleukemic syndrome characterized by clonal hematopoietic stem-cell disorders and peripheral cytopenia. In 1984, Kerndrup et al., noticed that the decreased NK activity seen in patients with MDS was caused by a decreased number of circulating NK cells [5]. In 1994, Yokose et al., correlated the decreased absolute number of CD3−CD16+ and CD3−CD56+ cells, in patients that had a high risk of MDS, with an increased plasmatic level of sIL-2R [11]. The authors hypothesized that plasma sIL-2R is produced by malignant MDS cells in the bone marrow and could impair IL-2 stimulated growth of NK cells. Nonetheless, an increased absolute number of NK cells can be associated with impaired cytotoxicity (Sanchez et al., personal data). Thus, in most hematological malignancies, qualitative impairment of the capacity of NK cytotoxic seems more important for tumor escape than quantitative defects.

3. Tumor Escape from NK Cell Surveillance: Role of NK Cells Effectors' Qualitative Deficiency

3.1. NK Cell Qualitative Deficiency by Increased Inhibition of NK Cell Cytotoxicity. Decreased expression of HLA class I molecules is a way for tumor cells to escape specific T-cell surveillance [35, 36]. Interestingly, the downregulation of HLA class I may allow NK cell targeting of tumor cells. Nevertheless, some reports from acute myeloid leukemia (AML) show a normal expression of HLA class I that inhibits the potential action of NK cells and leads to NK cell anergy [12]. Upregulation of HLA-A, -C, and -E molecule surface expression was demonstrated in a drug-resistant leukemic cell line that was also resistant to NK cell cytotoxicity [13]. Demanet et al. showed, in leukemic cells from 24 patients with acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL), selective downregulation of HLA-A and HLA-Bw6 associated with HLA-Bw4 preservation, which provided an escape mechanism not only from T-cells but also from NK cell surveillance [14].

Similarly, in CLL cells, Maki et al. have demonstrated and increased the expression of tolerogenic HLA-G1, a class I molecule that engages NK cell inhibitory molecules. Blockade of HLA-G1 with a specific antibody in CLL samples increased their susceptibility to NK-mediated killing, demonstrating that HLA-G1 participates in protecting CLL cells from NK-mediated killing [15]. Beside leukemia, upregulation of HLA class I has been also described in multiple myeloma and lymphoma [16, 17]. High expression of HLA-I molecules is observed in late-stage multiple myeloma (MM) plasma cells, and confers protection from NK lysis, even though NK cells efficiently kill early-stage tumoral plasma cells in a NCR- and NKG2D-dependent pathways [17].

3.2. NK Cell Qualitative Deficiency by Impaired Activation of NK Cells. In AML, the downregulation of NCRs NKp30/NCR3 and NKp46/NCR1 is associated with decreased NK cell cytotoxicity [8, 18]. In sharp contrast with healthy donors, in most patients with AML, the majority of NK cells display low NCR surface density (NCR^{dull}). This phenotype correlates with their weak cytolytic activity against autologous leukemic cells, which cannot be reversed by monoclonal antibody-mediated disruption of HLA class I/KIR-receptor interaction. This phenotype is partially or totally reversed when patients achieve CR, suggesting that this phenotype is probably acquired by direct contact between leukemia blasts and NK cells [19]. In addition, NK cell activity correlates positively with the relapse-free survival

Table 1: Mechanisms of escape, receptor, and ligand involved and type of hematological malignancy.

Mechanisms of escape	Receptor in NK cells	Ligand in tumor cells	Hematological malignancy type	References
NK-cell quantitative deficiency			MDS	[5, 11]
Increased expression of inhibitory receptors		Upregulation of HLA class I	AML	[12–14]
			CLL	[14, 15]
			LAL	[14]
			MM	[16]
			Lymphoma	[17]
Decreased activation by decreased expression of activating receptor or their ligands	NKp30	NCR-ligand	AML	[8, 18–20]
	NKp46NKG2D		CLL	[21]
			AML	
			LGL	[8]
		sMICA and sMICB	CLL	[22]
			CML	[23]
			MM	
			ALL	[24]
	DNAM1	NKG2D on tumor cells	CMML	[25]
				[26]
	CD94/NKG2C		AML	
				[27]
	2B4/CD244		AML	
				[27]
	CD16		AML	
			MM	[27]
			MM	[28, 29]
				[28, 29]
Impaired NK cell differentiation signaling			CML	[30]
			PV	Personal data
Impaired cytokine production	Elevated TNF		MDS	[31]
	Elevated PDGF		MPS	[32]
	Elevated TGFb			[33]
	Decreased IL1		AML	[34]
	IL2 and IFNγ		ALL	[34]

Abbreviations: MDS: myelodysplastic syndrome; MPS: myeloproliferative syndrome; ALL: acute lymphoid leukemia; AML: acute myeloid leukemia; CML: chronic myeloid leukemia; MM: multiple myeloma; CMML: myelomonocytic leukemia; LGL: large granular lymphoma; PV: polycythemia vera; sMICA: stress-induced molecule HLA class-I chain-related A; IL: interleukin; IFNγ: interferon-gamma.

of patients with AML [7]. In CLL, although no difference is observed regarding NCR expression between patients and age-matched healthy controls, decreased NCR expression correlates with a poor prognosis [21]. Another mechanism that is pivotal to the immune escape of AML blasts, is the downregulation of ligands relevant in NCR-mediated target-cell recognition [8]. Together, insufficient activation, caused by defects in NCR expression and deficient expression of their ligands on leukemic target cells, may represent a potent method for tumor immune evasion. These abnormalities, when present at the diagnosis of leukemia, may also be related to a worse outcome [19].

Another activating receptor, NKG2D, has a crucial role in NK cell activation. The NKG2D ligands, MICA, and MICB [37], are expressed on half of AML blasts [38]. Leukemia cells that express MICA are lysed *in vitro* by NK cells via NKG2D engagement with the sera of patients, but not of healthy donors as the latter contain elevated levels of seric soluble MICA (sMICA), which impairs NKG2D-mediated immune surveillance of leukemia by triggering internalization of surface NKG2D [39]. In large granular lymphocyte (LGL) leukemia, the impaired cytolytic function of NK cells is associated with reduced expression of NKG2D, which correlates with disease progression [22]. Abnormally high levels of sMICA and weak expression

of NKG2D on NK cells have also been reported in chronic myeloid leukemia (CML) [40]. Interestingly, these abnormalities are reversed by imatinib mesylate therapy. In this setting, the level of MICA expression is under the posttranscriptional control of BCR/ABL tyrosine-kinase activity [40]. Similarly, MM patients have high levels of sMICA whereas tumor cells express low levels of MICA [24]. In contrast, in monoclonal gammopathy of undetermined significance (MGUS), a common disorder of aging and a precursor lesion to MM, tumor cells express high levels of MICA whereas low levels of sMICA are detected in peripheral blood. These data suggest that alterations of the NKG2D pathway are associated with progression from MGUS to MM [24]. Alteration of the NKG2D pathway is also observed in CLL and could, in part, be responsible for the defective NK cytotoxicity observed [15]. CLL leukemic cells do not express MICA- or UL-16-binding proteins (ULBPs) (excepted a very low expression of ULB3) [23]. Similarly, NK-resistant B-ALLs cells do not express MICA, MICB [25], or ULBPs (or only at very low levels) [41].

Recently, Weiss-Steider et al. demonstrated that myelomonocytic leukemia cells produce and secrete MICA and MICB, but also express the receptor NKG2D, resulting in an *in situ* depletion of stress signals, thus avoiding activation of NK cells [26]. Moreover, MICA and MICB appeared to act as a tumor-growth factor resulting in strong tumor proliferation in dose-dependent induction [26].

In addition to the downregulation of NCRs and NKG2D, downregulation of the NK-cell activating receptors or the co-receptor DNAM-1, 2B4/CD244 and CD94/NKG2C have been described in AML [38]. Similar to the NKG2D pathway, chronic exposure of these activating molecules to their ligands may be responsible for their downregulation [27]. This has been demonstrated for DNAM-1 since an inverse correlation between DNAM-1-ligand expression on leukemic blasts and DNAM-1 expression on NK cells has been found. Furthermore, the culture of NK cells from healthy donors with leukemic blasts that express DNAM-1 ligands induce DNAM-1 downregulation at the NK cell surface [27]. NK cell costimulatory molecule 2B4/CD244 has a natural ligand that is CD48, a molecule that is highly expressed in hematological malignancy cells [42]. In MM, despite normal NCR and NKG2D expression [17, 28, 29], there is drastic downregulation of CD16 and 2B4/CD244, which leads to decreased ADCC in MM [28, 29]. The downregulation of CD16 can be explain by the continuous exposure to circulating monoclonal immunoglobulin.

3.3. NK Cell Qualitative Deficiency by Impaired Differentiation Signaling. Altered NK differentiation from CML CD34+ progenitors is linked to their constitutive production of bioactive IL-15, which does not lead to NK cell differentiation [43]. This has been directly attributed to the *BCR-ABL* mutation, because the addition of BCR-ABL-transfected stem cells suppresses the differentiation of autologous normal cord blood CD34+CD38− [30]. Impairment of the signaling pathway is also suspected in polycythemia vera (PV), an MP disorder associated with an acquired activated mutation of the tyrosin kinase JAK2. Patients with PV have an increased percentage and absolute number of NK cells with impaired cytotoxic functions, not caused by variation in inhibitory or activating receptors (Sanchez et al., personal data). The NK cytotoxic defect may be related to the direct or indirect impairment of cytotoxicity signaling pathways, putatively linked to JAK2 mutation. We are currently testing this hypothesis via the transcriptomic study of NK cells taken from PV patients (Baier et al., personal data).

4. Tumor Escape from NK Cell Surveillance: The Pivotal Role of Cytokines

Several cytokines are known to inhibit NK cell activation. In 1992, Verhoef et al. suggested that elevated and circulating levels of tumor-necrosis factor (TNF) impairs NK cytotoxicity in MDS [31]. Similarly, the high levels of PDGF, detected in myeloproliferative syndrome (MPS) could impair NK cell cytotoxicity [32]. TGF-β has also been implied in the inhibition of NK cell function following chronic interaction with tumor cells [33]. Indeed TGF-β antagonizes IL-15, which induces proliferation and gene expression associated with NK cell activation, resulting in inhibition of both NK cell activating receptor molecules and components of the cytotoxic apparatus [33]. Impaired NK cell function as been also linked to a decrease production of NK cell activating cytokine such as IL-1, interferon-gamma and IL-2 in the peripheral blood mononuclear cells of AML and ALL patients [34].

5. Therapeutics Implications "from Bench to Bedside"

As chemotherapy can disrupt potentially competent immune-surveillance mechanisms, thus favoring a relapse via induced immune suppression, the necessity of developing immunotherapy that enhances immune surveillance after chemotherapy is discontinued is admitted [44]. Markasz et al. have characterized the effect of 28 frequently used chemotherapeutic agents on the capacity of NK cells to kill tumor cells [45]. Although most chemotherapy drugs quantitatively decrease NK cell counts, some inhibit NK-cell activity whereas others enhance NK cells ability to kill tumors cells [45].

The greatest revelation of NK cells role in tumor control has been shown in allogenic transplantation. In 2002, Ruggeri et al. demonstrated a favorable prognosis for AML patients who were recipients of a haploidentical allogenic transplant with a NK HLA-specific receptor mismatch [46]: this enhanced the antileukemic graft reactivity. Interestingly, NK cell mismatch provides a graft-versus-leukemia (GvL) effect that is devoid of the deleterious graft-versus-host (GvH) reaction. This is because host cells, apart from leukemia cells, do not express activating ligands for NK cell cytotoxicity [47, 48]. Conversely, the benefit of NK-cell alloreactivity after unrelated cord-blood stem-cell transplantation remains unclear and is controversial [49]. Miller

Table 2: Novel agents used in hematological malignancies and their impact on NK-cell activation.

Class of drugs	Therapeutic molecules	Main indications	Inhibitory receptor ligands	Activating receptors	Activating receptor ligands	References
IMIDs	Thalidomide Lenalidomide	Multiple myeloma		NKp46 upregulation		[67]
HDACI	Vorinostat Panobinostat	Lymphoma		NKp30 and NKp46 downregulation	NKG2D and DNAM ligand upregulation	[68, 69]
Demethylating agents	5-azacytidine 5-aza-2′-deoxycytidine	MDS	Up regulation KIR		NKG2D ligand upregulation	[70, 71]
Proteasome inhibitors	Bortezomib	Multiple myeloma	Downregulation HLA molecules		NKG2D TRAIL and DNAM ligand upregulation	[72–75]
All-trans retinoic acid	Vesanoid	AML3		NKG2D Upregulation		[76]
Tyrosine kinase inhibitors	Imatinib mesylate	CML		NKG2D upregulation	sMICA downregulation	[77]

Abbreviations: IMIDs: immunomodulaory drugs; HDACI: Histone deacetylase inhibitors; MDS: myelodysplastic syndrome; AML: acute myeloid leukemia; CML: chronic myeloid leukemia; NK: natural killer cell; sMICA: stress-induced molecule HLA class-I chain-related A.

et al. were the first to infuse NK cells from haploidentical origins into AML recipients in a nontransplant setting [50]. Haploidentical NK cells can persist and expand *in vivo* if patients receive intensive immunosuppression regimens before NK cell infusion [50]. Successful transfer of alloreactive haploidentical KIR ligand-mismatched NK cells was performed in 13 elderly patients after administration of a combined immunosuppressive therapy of fludarabine plus cyclophosphamide, followed by subcutaneous administration of IL-2 for 2 weeks. Donor *versus* recipient alloreactive NK cells were shown to kill recipient leukemia cells. This phase I study demonstrates that infusion of purified NK cells is feasible and safe [51]. A phase II study, using a greater dose of NK cells and multiple infusions is being currently conducted. Similarly, a pilot study on transfer of alloreactive haploidentical KIR ligand-mismatched NK cells has been performed in children with AML after achievement of CR. The study gave encouraging results, and has allowed commencement of a phase II trial as consolidation therapy in children with AML [52]. In MM myeloma, infusion of haploidentical KIR-ligand mismatched NK cells has been proposed in relapsed patients in the setting of autologous stem-cell transplantation [53].

Since allogeneic transplantation raises several problems regarding high morbidity and mortality rates, manipulation of autologous NK cells rises increasing interest. The IL-15 supports large-scale expansion of NK cells by preserving activating-receptor expression [54]. *In vivo*, the TGF-β blockade, by both anti-TGF antibodies and a small molecule inhibitor of TGF-β signaling, is an interesting way to suppress the NK-cytotoxicity inhibition caused by TGF-β release by tumors cells [33]. In addition, because interferon (IFN)α enhances IFNγ and IL-12 secretion, which in turn triggers NK cytotoxicity, Lion et al. proposed IFNα post-CR maintenance immunotherapy for AML patients [55].

Another therapeutic approach to enhance NK cell cytotoxicity is the use of cytokines, such as IL-2, IL12, and IL18 alone, or in association with histamine dihydrochloride, which enhance both NK- and T-lymphocyte proliferation and cytotoxicity [56, 57]. In AML, a postconsolidation regimen with an association between histamine dihydrochloride and IL-2 has resulted in improved progression-free survival (PFS) for patients (at 3 years PFS 40% *versus* 26% for controls) [58]. This association protected NK from the downregulation of activating-receptor expression (more particularly NKp46/NCR1) induced by leukemia cells; thus, it enhanced NK cytotoxicity and tumor immune control with significant improvement in leukemia-free survival [59].

Chemokine manipulation is also of interest as molecules may both attract NK to the tumor-cell microenvironment and stimulate their cytotoxic properties [60]. Specific chemokine inhibitors are currently under investigation, although redundancy and pleiotropy of the chemokine system are obstacles in drug development [61]. An alternative immunotherapy based on cytokine manipulation is NK stimulation via dendritic cells (DCs). Stimulation of DCs using TLR9 agonists converts tolerogenic DCs into immunogenic DCs, and allows DC activation and cytokine secretion, which enhances NK cytotoxicity [62].

Another attractive approach to enhance NK cytotoxicity is to use monoclonal antibodies (mAbs). IPH-2101, a fully human IgG4 anti-KIR mAb (developed by Innate Pharma) is currently being tested in phase I and II clinical trials in patients with AML and MM [63]. Its blockade of inhibition could allow NK-cell activation when activating ligands are present on target cells. Preliminary results show enhanced NK cell activity has a good safety profile [63]. Bispecific mAbs directed against both the target cells and cytotoxic effectors (NK cells) are also currently under investigation. Anti-CD20 mAbs that have enhanced affinity for CD16

have been also developed, and they are more effective at NK activation than rituximab [64, 65]. Similarly, bispecific mAbs, which targets the CD16 molecule on the NK cell surface and CD30 (the molecule expressed in Hodgkin's tumor and anaplastic T-cell lymphoma), are currently being evaluated [66].

Novel drugs with immunomodulatory properties are increasingly being used these days to treat hematological malignancies. Most have an impact on NK cell activity and/or target susceptibility to NK lysis. Table 2 briefly outlines the major mechanisms identified or suspected that could explain their impact on NK cell cytotoxicity (see [9]).

6. Conclusion

As the knowledge of NK cells' role in tumor surveillance increases, therapies to boost NK immunity have emerged. Because impairment of NK cell cytotoxicity is associated with almost all hematological malignancies, restoration of normal NK function is an attractive goal for immunotherapy. New approaches attempt to boost immune surveillance by enhancing NK cytotoxicity and tumor-cell susceptibility to NK lysis. Because the mechanisms that tumors use to escape NK surveillance are multiple, there are multiple potential ways to increase the NK cell lysis of tumor cells, that is, by increasing expression of activating receptors, diminishing or counteracting expression of inhibitory receptors, increasing NK cell cytotoxicity, and modulating target-cell sensibility to NK cell lysis via upregulation of ligand expression on the tumor-cell's surface. Combined strategies, including cytotoxic conventional chemotherapy with immunomodulatory agents, and NK cell manipulation, are becoming increasingly attractive.

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Review Article

Lung Cancer: A Classic Example of Tumor Escape and Progression While Providing Opportunities for Immunological Intervention

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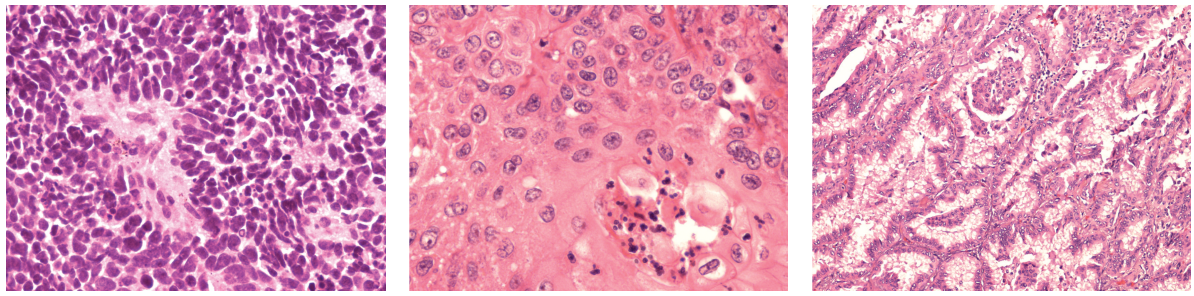
Lung cancers remain one of the most common and deadly cancers in the world today (12.5% of newly diagnosed cancers) despite current advances in chemo- and radiation therapies. Often, by the time these tumors are diagnosed, they have already metastasized. These tumors demonstrate the classic hallmarks of cancer in that they have advanced defensive strategies allowing them to escape various standard oncological treatments. Immunotherapy is making inroads towards effectively treating other fatal cancers, such as melanoma, glioblastoma multiforme, and castrate-resistant prostate cancers. This paper will cover the escape mechanisms of bronchogenic lung cancer that must be overcome before they can be successfully treated. We also review the history of immunotherapy directed towards lung cancers.

1. Introduction

Approximately 12.5% of the newly diagnosed cancers in the world are lung cancers (World Cancer Research Foundation International). Lung cancer leads the world in newly diagnosed cancers: 1.6 million new cases were diagnosed in 2008. In the USA, about 225,000 newly diagnosed patients are annually reported. Bronchogenic lung cancers (LCs) have very fast growth rates. This basic aspect of lung cancer biology makes them sensitive to chemo- and radiation-based therapies for a temporary palliative treatment. These treated lung tumors will eventually relapse because a number of cancer clones or "cancer initiating cells" have escaped the initial therapy. These cells are selected and will return with enhanced resistance to therapeutic modalities. Additional adjuvant treatments are needed to eliminate those remaining cells that survived the initial therapy. Immunotherapy provides the opportunity to destroy the residual lung cancer cells that chemotherapy and radiation miss and may attack the so-called "cancer stem cells." By activating the host immune lymphocytes, these cells can theoretically infiltrate into those remaining pockets of tumor cells and eliminate them. Progress has been made using immunotherapy to successfully improve the survival of some patients with other fatal types of cancer, such as glioblastoma multiforme and castrate-resistant prostate cancer [1, 2]. Some of the lessons learned from those cancers can be directly applied to cure lung cancer, too.

2. Derivations of Lung Cancer

Lung cancers (LCs) are very aggressive tumors derived from different cell types. The incidence of LC in the western world rose precipitously during the 20th century due to increased prevalence of smoking. The prevalence of new lung cancer is dropping in those western countries that



(a) (b) (c)

Figure 1: Representative histological micrographs of the most common lung cancers. (a) Small-cell lung cancer taken with a 40x objective lens. (b) Squamous cell lung cancer visualized with a 40x objective lens. (c) Adenocarcinoma lung cancer seen using a 10x objective lens.

successfully discourage smoking; however, the incidence of LC is now rising in those developing countries that see smoking as an easy form of tax revenues. These smoking-induced lung cancers are predominantly caused by Ras mutations. Tobacco smoke contains many carcinogens [3] including very complex aromatic hydrocarbons (benzopyrenes) and other organic compounds such as nitrosamines. The classic example of cancer induction due to chronic carcinogenic exposure usually involves tobacco-derived carcinogens. Once these carcinogens enter healthy cells they induce genetic mutations, which lead to oncogenic transformation. Besides chemical carcinogens, asbestos, radioactive radon, polonium, and plutonium can also contribute to the formation of LC. Finally, there are individuals who can spontaneously develop lung cancer without any known carcinogenic exposures. Asian women, usually of Japanese descent, possess epidermal growth factor receptor (EGFR) mutations [4, 5]. Some younger men have an echinoderm microtubule-associated protein-like 4 (EML4) and anaplastic lymphoma kinase (ALK) translocation (EML4-ALK) [6, 7], which causes their cancer.

Small-cell lung cancer (SCLC) arises from neuroendocrine cells, the "Kulchitsky cells" of the lungs. These cancers represent about 20–30% of all lung cancer diagnoses in the USA. This type of lung cancer was previously called "oat cell" cancer. These cells make polypeptide hormones and are characterized by dense core neurosecretory granules. These small-cell lung cancers are different from the non-small-cell lung cancers (NSCLCs). NSCLC includes the adenocarcinomas, squamous and large cell cancers usually arise from alveolar cells. Adenocarcinomas come from basal bronchial cells and type II pneumocytes that arise in the periphery of the lung, while the squamous type lung cancers arise from the bronchial epithelial cells located more centrally. The incidence of squamous lung cancer is dropping in the USA and it has now been overtaken by adenocarcinomas, perhaps due to the reformulation of cigarettes back in the 1970s to contain filters. These filters prevent the larger particulate matter containing the carcinogens from getting into the lungs. The smaller carcinogenic smoke particles still reach into the depths of the lungs. Thus, the percentage of non-small-cell lung cancers (NSCLCs) is now trending towards a more adenocarcinomous type of cancer. The two types of spontaneous lung cancers due to nonsmoking causes are predominantly adenocarcinomas. These types of cancers are thought to be a totally different type of lung cancer when compared to those adenocarcinomas generated by smoking [8].

3. Pathological Characteristics of Lung Cancers

Lung cancers are mostly bronchogenic carcinomas. Small-cell carcinoma consists of round, oval, and spindle-shaped malignant small cells with scant cytoplasm, ill-defined cell borders, and finely granular nuclear chromatin. Nuclear molding is prominent. Mitotic count is high and usually presents with extensive necrosis (Figure 1(a)). Squamous cell carcinoma is characterized by infiltrating nests of malignant epithelial cells with keratinization and/or intercellular bridges (Figure 1(b)). Adenocarcinoma typically shows glandular differentiation with acinar, papillary, bronchioalveolar, solid, or mixed pattern with mucin production (Figure 1(c)). Ruffini and coworkers [9] presented data which showed that *in situ* adenocarcinomas were infiltrated by lymphocytes about 25% of the time. In contrast, only 5% of SCLCs had lymphocytic infiltrates. SCLCs have a higher proliferative rate than the adenocarcinomas.

4. Survival of Lung Cancer Patients

Because lung cancer cells divide so rapidly, they are temporarily treatable by standard oncological therapies. Localized radiation is applied if the cancer is still physically found within the affected regions of the lung. Adenocarcinomas and squamous cell carcinomas are usually surgically removed as a first step. By the time small-cell lung cancers are discovered they have usually disseminated systemically and so tend to have a poorer prognosis when compared to early detection of NSCLC. Systemic chemotherapy after surgical resection is given. Localized LCs have the best prognoses, whereas metastatic LCs have the worst survival. Lung cancer patients have an overall 5-year survival rate of 16% (2011 American Cancer Society). The survival statistics indicate that these tumor cells have a variety of escape mechanisms that counteract current therapeutic interventions. These escape pathways include antiapoptosis, drug resistance, and immunodefensive routes. Table 1 shows the various escape Pathways that lung cancers can use and will be discussed in detail.

Table 1: Mechanisms of lung tumor escape.

(1) Antiapoptosis genes
(a) Bcl-2, survivin
(b) Loss of apoptosis effector molecules: caspases, p53 family
(2) Drug resistance genes
(a) Multidrug resistance proteins
(b) CD133
(3) Immunoresistance genes
(a) Soluble factors: PGE, VEGF, TGF-β, Ido-1, arginase
(b) Immunosuppressive cells: Treg and MDSC
(c) Loss of classical MHC and/or gain of nonconventional MHC
(d) Counterattack: Fas ligand and PD-L/B7-H family members
(e) Age and loss of functional immune system

5. Escape Mechanisms

5.1. Antiapoptosis Genes. Radiation and most chemotherapeutic drugs kill tumor cells via apoptosis. Many "cancer stem-like" cells are radio- and chemotherapeutic resistant [10–13]. Lagadec and coworkers [14] showed with breast cancer stem cells that upon radiation treatment reprograms those remaining cells. So when these cancers relapse they will come back with enhanced antiapoptotic gene expression. These altered profiles include increased bcl2, survivin, and livin, which makes these relapsing cancer cells more resistant to drugs that were previously used to treat the initial LC [15–22]. Bcl2 is highly expressed in SCLCs and somewhat less expressed in squamous lung cancers (about one third as much as found in SCLCs), [23]. Survivin is highly expressed in most NSCLCs [21]. These mutations make the cancer cells more resistant to therapy when treatments are given. Bcl2 and survivin are both induced by PGE_2 [24, 25] (see Section 5.3.1(a)). Many of these genetic alterations and mutations within LC have been directly attributed to the actions of carcinogens. Joseph et al. observed that SCLC lost production of caspase-1, -4, -8, and -10 (apoptosis effector molecules) [26]. It has been postulated that gene silencing via altered methylation profiles [27, 28] might be responsible for some of this loss of apoptosis executioner proteins within the SCLCs.

Another type of mutation that frequently occurs within lung cancers would be point mutations within the p53 suppressor gene. P53 mutations within lung cancer lead to upregulation of Bcl2 while downregulating the pro-apoptotic expression of Bax [29]. Zöchbauer-Müller et al. [30] have reported that SCLCs and NSCLCs both contain these p53 mutations. There are other members of this family (e.g., p63, p73) [31, 32] that perform similar functions as p53. Mutations in these p53 family members prevent apoptosis execution functions, too. Consequently, these mutations prevent tumor cells from killing themselves in response to various therapies.

Apoptosis is called the "silent death." When cells die of classic apoptotic pathways, these dead apoptotic cells tend to be rapidly absorbed or "scavenged" by the adjacent cells. This prevents the local antigen-presenting cells (APCs) from having sufficient time to absorb this cellular debris in order to stimulate the immune system. In contrast, when cells die of a necrosis-dependent pathway, the immune system is now activated and begins an active immune response in response to the "danger signals" that have been released by the necrotic cells [33–37]. Danger signals include high gel mobility box-1 (HMGB1), uric acid, calreticulin, and the heat shock proteins (HSPs) [38]. Hence, drugs that kill tumor cells via this necrosis induction pathway provide better long-term effects by enhancing the immune system response to the cancer as they regress. There are cytotoxic drugs that can kill tumor cells via apoptosis and stimulate immune responses. A more appropriate terminology has been coined: "immunogenic" versus "nonimmunogenic" cell death to better represent the more nuanced version of this phenomenon [39, 40]. The judicious use of chemotherapeutic drugs that promote "immunogenic" apoptotic death may further improve chemotherapy against lung cancers by stimulating endogenous immune responses against the tumors.

5.2. Drug Resistance Genes. Another strategy that tumor cells use to evade the full effects of cytotoxic drugs is to export the chemotherapeutic drugs from the cells. Newly synthesized drug-resistant transmembrane proteins actively pump out the chemotherapeutic drugs that have entered into the cancer cell. These exporters effectively reduce the internal concentration of the drug, thereby preventing the full cytotoxic effects of the drug. The small amount of the drugs that remain may even activate cell repair mechanisms such as the cell stress pathways, which would allow heat shock proteins to remove and replace any damaged cellular components. This process may further promote drug resistance by the tumor cells. Lung cancers produce a variety of multidrug resistance proteins (MRPs) and P-glycoprotein [41–43]. In studies by Triller et al. [44] it was shown that when SCLCs relapse there are higher concentrations of MRP3 within the returning cancer cells.

CD133 has been reported to be a marker for various cancer stem cells, including NSCLC [12], although there is a report that their presumed NSCLC stem cells are aldehyde

dehydrogenase-positive cells [45]. For brain cancers, CD133 is a marker of bioenergetic stress [46] and probably reflects the universal function of this molecule with many different cancer types. CD133 was initially described as a fluorescent dye reverse transporter [13, 47, 48]. Many fluorescent dyes have planar chemical structures that resemble many chemotherapeutic drugs. Therefore, if CD133+ cells are able to export chemotherapeutic drugs via this proposed mechanism, then these stem cells are naturally resistant to chemotherapy. Bertolini and colleagues [12] showed that CD133+ lung cancer cells resisted cytotoxic doses of cisplatinum. By asymmetric division, CD133+ "stem cells" differentiate into CD133-negative cancer cells. These CD133-negative cells are subsequently more likely to be killed by chemotherapy. Many early cancer treatments seem to be effective at first, with the bulk of the tumor disappearing. But over time the tumor returns. Thus, the new explanation is that "stem cells" are drug-resistant clones that manage to escape treatment and are responsible for reestablishing the tumor, after the therapy has stopped.

5.3. Immunoresistance Pathways. Lung cancers produce numerous defense strategies that allow them to hide from the immune system. These pathways include releasing soluble immunosuppressive agents, recruitment of suppressor-type cells, lack of immune stimulating molecules, and counterattack strategies.

5.3.1. Soluble Immunosuppressive Mediators. These mediators include prostaglandin (PGE) via cyclo-oxygenase, interleukin-10, other types of type-2 derived cytokines, transforming growth factor-β, and vascular endothelial growth factor, which inhibit *in situ* immune responses. These soluble mediators work in a variety of ways and most likely synergize with one other.

(a) Prostaglandin. Lung cancers produce several types of prostanoids and leukotrienes [49, 50], which are derived from arachidonic acid metabolism. Normal lung cells make little to no prostaglandin, whereas lung cancers (squamous, adenocarcinoma, small cell, and mixed lung cancers) produce elevated levels of prostaglandin E_2 (PGE_2). PGE_2 is synthesized by either cyclo-oxygenase-1 (Cox-1) or -2 (Cox-2). Cox-1 is a constitutive enzyme that produces low levels of PGE_2, while Cox-2 is the inducible form of the enzyme and produces much more PGE_2. Many tumors [51], including lung cancers [52], overexpress the Cox-2 enzyme. Epidemiological studies have shown that a daily dose of aspirin helps reduce PGE_2 production and lowers the incidence of a variety of cancers including lung cancers [53]. Prostaglandin E_2 can bind to the 4 different prostaglandin receptors: EP1, EP2, EP3, and EP4 [54]. Lung cancer cell lines and *in situ* lung cancers express a variety of these receptors [55]. PGE_2 regulates several aspects of lung cancer biology such as controlling angiogenesis, ERK stimulation, invasion, and proliferation [56–59]. PGE_2 raises intracellular cyclic AMP levels [60, 61] within various immunocytes, which inhibits dendritic cells and prevents B, T, and NK cell effector functions.

(b) Interleukin-10. PGE_2 also stimulates IL-10 production from a variety of cell types, macrophages, B cells, and T cells [62, 63]. Current dogma holds that interleukin 10 is a Th2 cytokine. IL-10 downregulates Th1 immunity (see below) and prevents effective antitumor immunity [64]. But there is a body of evidence which shows that IL-10 can actually assist in antitumor immune responses in a variety of tumor models, including lung cancer [65–67]. Thus, the role of IL-10 in tumor rejection is not as clear cut as previously thought and may be a double-edged sword. IL-10 may work in concert with other immunosuppressive agents or suppressor cells to produce protumor effects. LCs are known to produce Th2 polarization by releasing cytokines, IL-4, IL-5, IL-6, and IL-13, besides IL-10 [68–70]. These other cytokines along with PGE_2 could either simply mask the effects of IL-10 or synergize with IL-10 to enhance the protumor activities. So a word of caution needs to be applied with IL-10.

(c) Vascular Endothelial Cell Growth Factor. All types of cancers, including lung cancers, make vascular endothelial cell growth factor (VEGF) [71–74]. Some LCs also express the VEGF receptors, suggesting that autocrine loops may control tumor cell growth [73, 75, 76]. VEGF promotes tumor angiogenesis by recruiting endothelial precursor cells from the blood to begin building new blood capillaries. These new blood vessels supply the tumor with oxygen and nutrients allowing the tumor to grow. Vascular permeability functions of endothelial cells are also enhanced by VEGF. VEGF also provides a mechanism by which the immune system is inhibited, by downregulating the functions of antigen-presenting cells (APCs) [77], adaptive immune responses are thereby prevented. The VEGF gene within the general population has several polymorphisms [78]. These polymorphisms may make certain individuals more susceptible to developing lung cancer and may explain why not all smokers develop lung cancer.

(d) Transforming Growth Factor-β. Transforming growth factor-β (TGF-β) is another commonly overexpressed cytokine that performs multiple functions in tumor biology [79–81]. These activities include assisting tumor growth, improving angiogenesis, enhanced migration, fibrosis production, and increased proteolytic enzyme release, while simultaneously inhibiting the immune response. Lung cancers do overexpress several TGF-β isoforms-1, -2, and -3 while concurrently having mutated TGF-β receptors [82–88]. These mutations within the receptors prevent the negative signaling transduction pathways being delivered by TGF-β to the tumor cells. Simultaneously, this excess TGF-β influences the local microenvironment. TGF-β is a well known factor and is very good at inhibiting many aspects of cellular immunity (reviewed in [89, 90]). Like VEGF, there are reported polymorphisms in the TFG-β1 that make certain individual less susceptible to developing lung cancer [91].

5.3.2. Immune Suppressor Cells. Many of these immunosuppressive agents listed above will recruit either Treg [91] or myeloid derived suppressor cells (MDSCs) [92]. The combined milieu of all of these immunosuppressive

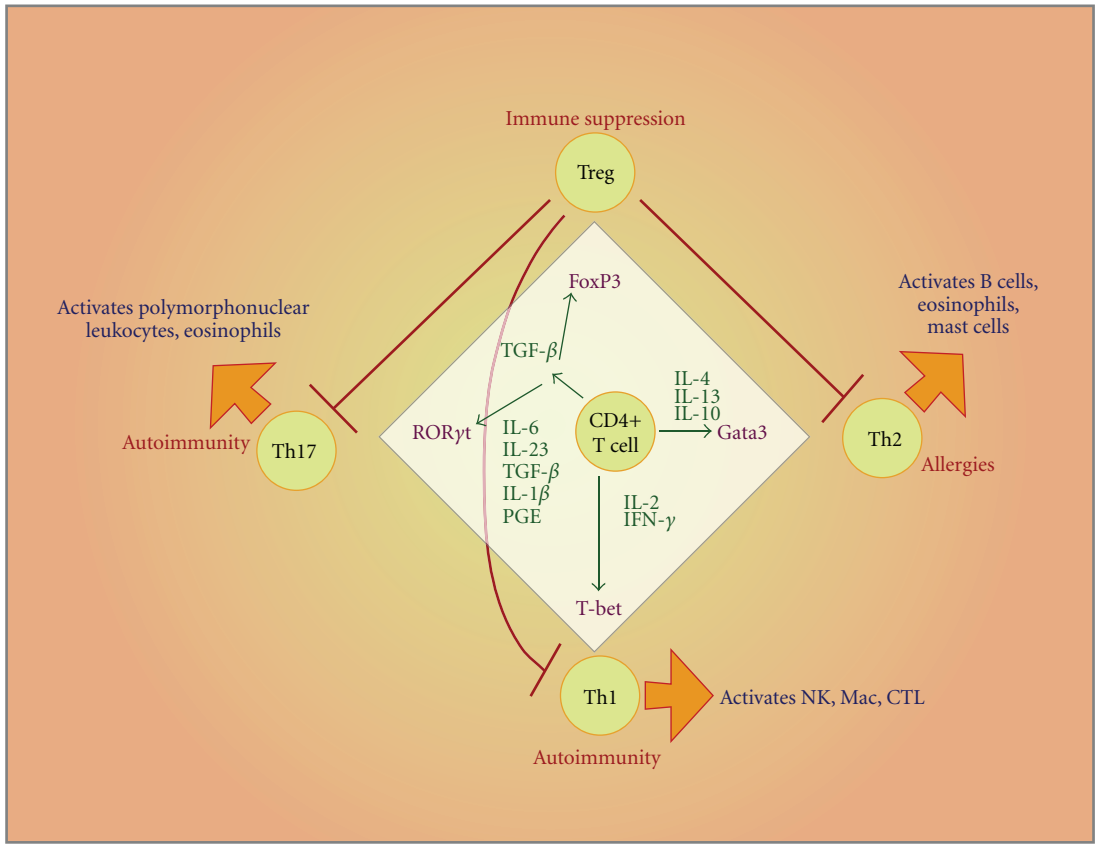


Figure 2: The four different types of CD4+ T cells. The different types of CD4 T cells start from common precursor T cells. Upon stimulation with the different cytokines, the naïve CD4+ cells now get to be selected by various transcription factors. Th1 cells become polarized towards this phenotype in response to IL-2 and IFN-γ, and the transcription factor, T-bet, now controls the fate of these cells. In response to IL-4 or IL-13, the Gata3 transcription factor becomes active and Th2 cells result. TGF-β now stimulates a common Th17/Treg cell. Upon stimulation with IL-6, IL-23, TGF-β, IL-1β (in humans), and PGE, Th17 cells become activated through a RORγT transcription factor. Tregs become polarized by FoxP3. The Tregs have the ability to inhibit Th1, Th2, and Th17 black arrows. The red arrows indicate the effector functions of the various CD4+ subsets. The side effects autoimmunity, allergies, or immune suppression are also noted.

agents can bring about proper conditions that allow these suppressor cells to become the dominant immunologically active cells within lung cancers. The role of these types of suppressor cells is thought to be as a fail-safe mechanism by which the immune system is tightly regulated to prevent autoimmunity or other self-destruction. Lung cancer cells take full advantage of these suppressor cells.

(a) T-Cell Biology. CD4+ T cells can currently be classified into at least 4 different types and each has its own unique function (Figure 2). The different types of CD4 T cells start from common precursor T cells (so-called naïve or ThP cells). Upon stimulation with the different cytokines, the naïve CD4+ cells are selected by various transcription factors in response to various cytokines or other mediators. Th1 cells become polarized towards this phenotype in response to IL-2 and IFN-γ and the transcription factor, T-bet, now controls the fate of these cells. These Th1 cells upon activation will release other cytokines that activate cell-mediated effector functions such as CTLs, NK, and macrophages. These effector cells then eliminate cells infected with either intracellular bacteria or viruses. Th1 cells are believed to play major roles in fighting tumors. In response to IL-4, IL-10 or IL-13, the Gata3 transcription factor becomes active and Th2 cells differentiate. Th2 cells activate B cells so that they can make more high-affinity antibodies to help control extracellular bacteria and viruses. The high antibody titers made in response to prophylactic vaccination against these extracellular pathogens are usually attributed to the actions of Th2 cells. Increased IL-4 and IL-5 secretion by Th2 cells stimulates the B cells into producing IgE antibody. IgE in turn causes allergies like hay fever. IgE plays an effector role in fighting large extracellular parasites such as helminths.

TGF-β stimulates a common Th17/Treg cell. Upon stimulation with IL-6, IL-23, TGF-β, IL-1β (in humans), and PGE [93, 94], Th17 cells become activated through a RORγT transcription factor. Upon activation, Th17 cells play an inflammatory role like Th1 but tend to recruit myeloid cells such as neutrophils, monocytes, and macrophages as their effector cells. These myeloid effector cells control extracellular bacteria, parasites, and fungi. Overactive Th17 cells mediate certain autoimmune conditions. Tregs form as a result of the transcription factor, FoxP3, becoming the dominant transcription factor. The Tregs have the ability to

inhibit the actions of Th1, Th2, and Th17 cells. Tregs are essentially brakes that are thought to inhibit an overactive immune response towards any given antigen. Thus, Treg may be a way that produces immunological tolerance towards self.

(b) Th17 Cells. Th17 cells have been derived from a lung cancer patient who responded to Mage-A3 [95]. Prostaglandin E_2 regulates Th17 cell differentiation and if there is an increased amount of PGE_2 being produced then the presence of these Th17 cells in lung cancer can be explained [96, 97]. In a mouse model that has a metastatic melanoma of the lungs, Th17 cells promote CTL activity towards the cancer and help clear the tumor [98]. The role of Th17 in cancer biology is still not clear since there are reports that Th17 cells help promote cancer development (reviewed in [99]).

Both Th17 and Treg cells possess Aryl hydrocarbon receptors (AhRs) [100]. Activation of this receptor induces transcriptional regulation that controls these cells functions. Depending upon the exact hydrocarbons used, either Th17 or Treg cells can get activated with differing effects. Since tobacco smoke contains many different hydrocarbons, these receptors can easily activate both cell types. AhR activation on lung-derived fibroblasts also induce Cox-2 expression associated with increased PGE_2 production [101], so it is not surprising that an immunosuppressive environment is being established simply by the constant presence of these tobacco-derived hydrocarbons. AhR is expressed and used by human adenocarcinomas [102] to activate the CYP1B1. This P450 cytochrome enzyme converts noncarcinogens into carcinogens. Presumably, these same enzymes are activated within the precancerous cells that will be eventually turned into tumor cells [103]. AhR can also be found on dendritic cells, and upon activation these dendritic cells have the ability to express indoleamine 2′3′dioxygenase-1 (IDO-1) [104]. Ido-1 is known to inhibit immune responses (see below).

(c) Tregs. Th17 and Tregs share a common pathway, in that both require TGF-β for early development. IL-6 pushes the development of Th17 cells and IL-23 promotes the growth of already developed Th17 cells. In contrast, Tregs use IL-2 as a growth factor and possess the transcription factor, FoxP3, which drives their maturation and effector function. To eliminate these Treg cells, antibodies towards IL-2Rβ receptor or a recombinant IL-2-diphteria toxin fusion protein (Ontak, denileukin diftitox) [105] can be used to directly kill these cells and enhance immune responses towards tumors. Besides being an alkylating chemotherapeutic drug, cyclophosphamide also kills Treg cells. Tregs inhibit the immune system in part by possessing membrane TGF-β. Tregs release soluble TGF-β [106]. Treg are thought to be important in downregulating the Th1, Th2, and Th17 cells and perhaps preventing autoimmunity. Thus, it is not surprising that Tregs are found in abundance within lung cancers [107–110]. The increased presence of Treg is thought to explain why many therapeutic tumor vaccines do not work as well as they should in many cancer types.

Ido-1 is an enzyme specifically made by Treg. Ido-1 catabolizes the amino acid tryptophan. Here tryptophan is converted into kynurenine, which limits T cell responses, either because T cells require tryptophan to grow through mid-G1 arrest points or one of its metabolites inhibits T-cell-mediated functions [111–113]. The role of Ido-1 in immune escape mechanisms has been reviewed in Prendergast [114]. Some human lung cancers produce Ido-1, [115]. In *in situ* NSCLC, nine out of eleven cancers were Ido-1+ [113]. In a Lewis lung cancer model, Ido-1 was found to be made by the mononuclear cells infiltrating this tumor or by those cells present in the draining lymph nodes [116]. In human lung cancers, Ido-1 was being made by eosinophils that were infiltrating the NSCLC [117]. Ido-1 expression is strongly stimulated by IFN-γ [112]. This finding has significance because CTLs and NK cells could be releasing IFN-γ. This would inadvertently stimulate a homeostatic feedback loop that would deactivate the immune system via Ido-1. Ido-1 can be inhibited by using D-1-methyl tryptophan [118], an orally taken drug.

(d) Myeloid-Derived Suppressor Cells (MDSCs). Another type of suppressor cell is called the myeloid-derived suppressor cell. These cells have been recently reviewed by several groups [119–121]. These cells are derived from immature granulocytic or monocytic cells. Some of these cells are stimulated by interelukin-3 (IL-3, [122]), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), or PGE_2 [123]. Unlike Treg cells, which can be specifically targeted, MDSCs cannot be controlled as effectively since MDSCs are immature myeloid progenitor cells derived from normal hematopoiesis. Drugs are nevertheless being developed to inhibit MSDCs (reviewed in [124]). In a mouse colon cancer model, use of Cox-2 inhibitors can reduce the negative effects of immature MSDCs [125]. Brandau et al. [126] have shown the increased presence of MDSCs in NSCLCs. These MDSCs release enzymes called arginase [127, 128]. Arginase is a family of enzymes that catalyze the breakdown of arginine into ornithine and urea [129]. Arginase 1 is a cytoplasmic enzyme, whereas arginase 2 is a mitochondrial-derived enzyme. Arginine is needed for proper T-cell function and limits the ability of T cells to respond towards various antigens. Arginine effectively anergizes the T cells thereby making them tolerant towards their environment. Arginine controls immune responses in two opposing ways [130]. Nitric oxide synthase converts arginine into nitric oxide, and this polarizes T cells, DCs, and macrophages into a cell-mediated (Type 1) pathway. PGE_2 induces macrophages into producing arginase 1, polarizes macrophages into Type 2 cells, and inhibits cell-mediated Th1 immune responses [131]. Additionally, some MDSCs can also produce PGE_2 [132] as the way they suppress immune responses.

5.3.3. Downregulated Major Histocompatibility Complex (MHC) Makes Lung Cancers Invisible to the T Cells. Lung cancer cells can downmodulate their MHC antigen expression [133–135]. Many lung cancers express very little classic MHC molecules such as HLA-A, -B, or -C. Therefore, CD8 T cells are unable to recognize any tumor antigens that the cancer cells are expressing in the context of MHC class 1 molecules. This also explains why LCs are rarely

infiltrated by lymphoid cells [9]. Several pathways have been proposed to explain this loss of MHC expression. Loss of $\beta 2$ microglobulin and loss of transporter of antigen presentation (TAP) molecules [136–138] so that tumor peptides are not loaded successfully onto the MHC are two possible mechanisms to explain this defect. This defect can be corrected by adding cytokines like IFN-γ to these cells [139–141]. Thus, once activated Th1 cells can infiltrate the *in situ* tumor and produce IFN-γ [141–144]. This released cytokine may fully restore the expression of MHC on the lung cancer cells. However, when good MHC levels are present, beneficial antitumor responses are seen [145].

5.3.4. Increased Expression on Nonclassic MHC: HLA-E, HLA-F, and HLA-G. Tumors frequently express nonconventional MHC alleles such as HLA-E, HLA-F, and HLA-G (reviewed in [146, 147]). The exact role of these nonconventional antigens in tumor biology/immunology of cancers is still unknown. These molecules play a major role in preventing immune rejection of developing fetuses during pregnancy. Cancers may also hijack these molecules as a defense against the immune system. It is thought these nonconventional MHCs produce inhibitory type signals to the CTL or NK and prevent immune responses from occurring. Currently, there are reports that HLA-F and -G are expressed by various lung cancers [148–152]. Soluble HLA-G is reported to prevent proper antigen presenting function [153, 154]. The presence of HLA-E or -G now allows another type of T cells called the $\gamma\delta$ T cells the opportunity to counter this tumor defense strategy (see Section 7.5).

5.3.5. Counterattack. It is possible that tumor cells can express cell surface molecules, which have the ability to bind to receptors found on immunocytes and induce cell death or anergy. This process is called a counterattack.

Natural killer (NK) and cytotoxic T cells kill target cells via the release of soluble granzymes and perforin. Perforin essentially pokes holes into the target cells membrane and create an osmotic lysis. The released granzymes can enter these holes and bring enzymatic processes that ultimately result in target cell apoptosis. These cytolytic effector lymphocytes also express a membrane protein called Fas ligand. Fas ligand binds to another cell surface called Fas (also known as APO-l and CD95). CTLs can express Fas ligand (CD95L) and kill cells that are Fas+ [155], which can include lung cancers. Ligation of Fas by antibody induces apoptotic cell death in LC cell lines [156]. Niehans et al. [157] found that 16 of 16 human lung cancers (NSCLCs and SCLCs) expressed Fas ligand. Fas is found on many cell types, including T cells. Ligation of Fas by CTLs, NK or antibodies induces the FADD pathway that leads to caspase 8-dependent apoptosis within Fas+ cells. Two NSCLC cell lines (H2009 and H522) express FasL and have killed the Fas+ Jurkat T cells via a Fas-sensitive mediated cell death pathway [157]. Recently, this concept has been questioned [158]. But Fas ligand also recruits neutrophils into the lung cancers via the production of PGE_2 [159]. The recruited cells, including MSDCs, may partially explain the overall effect of the counterattack *in situ*. Thus, the exact role of FasL in lung cancer still needs to be fully identified.

One-third of primary lung cancers express a soluble decoy receptor, termed decoy receptor 3 (DcR3) [160]. This decoy binds to FasL and appears to inhibit FasL-mediated apoptosis. The fact that many LCs possess this decoy receptor suggests that Fas/Fas ligand must play an important role in lung cancer defense.

6. Impediments towards Lung Cancer Immunotherapy

Many arguments can be made against treating lung cancer with immunotherapy. Nonsmokers see this as a cancer that the smokers gave themselves due to their bad habit. Nonsmokers may consequently argue that developing immunotherapy for smoking-related lung cancers is a waste of time when there are many other types of nonsmoking-related cancers to treat. Hence, research funding is harder to procure for lung cancer. On a biological level, LCs have a variety of defense mechanisms: soluble mediators: transforming growth factor-β and cyclo-oxygenase-2, which makes prostaglandin E, interleukin-10, and arginase; defensive molecules such as Fas ligand, program death ligand-1 (B7-H1), nonconvention HLA molecules, lack of major histocompatibility (MHC) class I molecules, and recruitment of suppressor type cells. All these obstacles can naturally limit immune responses towards these cancer cells. These same arguments were also made against the immunotherapy of malignant human gliomas [161]. But progress is now being made against gliomas using dendritic cells pulsed with the patients' autologous cancers and other immunotherapies [1, 162, 163]. Thus, the development of immunotherapy towards lung cancer is significantly behind that observed with other types of immunotherapy for cancer.

7. Immunotherapy against Lung Cancers and Opportunities for Intervention

7.1. Early Steps. Since the beginnings of modern medicine, doctors have been looking for the "magic bullet" to treat tumors, whether it is through drugs, surgery, radiation, or other modalities. Back in the late nineteenth century William Coley discovered that a number of cancer patients who simultaneously had bacterial infections sometimes had miraculous cures to their cancers. He later used the bacteria isolated from those miraculous cures as toxins and his treatment was known as "Coley's toxins." Many explanations are possible, that is, released cytokines (IFN-γ, TNF), CpG segments of the bacterial DNA, and bacterial cell wall products, like LPS, can activate the toll-like receptors (TLRs) found on immunocytes, and so forth. Thus, biological response modifiers can enhance oncological therapies. Bacillus Calmette Guerin (BCG) was initially proved to be effective at treating superficial bladder cancer [164]. Injecting these bacteria into bladder cancers is now a routine therapy. This induced inflammation not only kills the tumor, probably by activating the innate immune system,

Table 2: Types of immunotherapy for lung cancer.

(1) Active vaccination: subunit vaccines from tumor lysates
(2) Passive antibody administration
(a) Direct tumor binding: various tumor antigens
(b) Indirect approaches: anti-VEGF, anti-CTLA-4 anti-PD-1/PD-L1, and anti-Treg
(3) Passive cell-mediated administration
(a) LAK cells
(b) TIL/draining lymph nodes T cells
(c) $\gamma\delta$ T cells
(4) Gene therapy
(a) IL-4
(b) GM-CSF
(c) MHC and/or costimulator molecules
(d) TGF-β knockdown
(5) Dendritic cell vaccination
(a) Peptides
(b) Tumor lysates

but also leads to sustained immune responses by activating the local antigen-presenting cells. BCG was tried to treat lung cancer but failed to show any responses [165–167]. Two other therapies such as using *Corynebacterium parvum* [168] and *Mycobacterium vaccae* [169] have been tried with lung cancers. Both of these therapies initially seemed to fail. However, when the data concerning the Mycobacteria vaccae was reevaluated, there was a higher survivor rate in patients who successfully completed the therapy than in those who did not [170]. Since compliance was a major issue with this therapy, it probably mean that many toxicities were occurring. The consequences of these toxicities probably means that it cannot be developed any further. Table 2 summarizes the various types of lung cancer immunotherapy that are currently being used to treat lung cancers.

Hollinshead et al. [171] showed significant 5-year survival rates for all types of lung cancer patients treated with a tumor antigen subunit vaccine purified from different types of lung cancers. The cancer vaccine was made by isolating the tumor antigens by an affinity chromatography technique using a variety of antibodies directed towards the antigens found on lung cancers. The 5-year survival of 234 treated patients with stage I and II patients was 69%, compared with 49% of the untreated controls. Unfortunately, this work did not proceed, despite its early promising success, since this vaccine was deemed dangerous. This vaccine was made with Freund's Complete Adjuvant (FCA), which contains mycobacterial proteins. These bacterial proteins stimulate the innate immune system via toll-like receptors (TLRs). The protocol included 3 vaccinations a month apart with FCA. FCA causes severe immune reactions and has painful reactions. Tissue damage such as ulceration is one potential sequela. These toxicities probably limited the further use of this promising protocol. This work did indicate that active immunotherapy can successfully treat some LC patients.

7.2. Direct Antibody Therapies. In the mid-1970s, Kohler and Milstein created hybridoma technology, which led to the creation of monoclonal antibodies. In the early to mid 1980s, this technology was commercialized for generating clinically applicable antibodies. Monoclonal antibodies were used to directly treat cancers early on. These antibodies were thought to be the "magic bullets" that could kill tumor cells. The advantage was that these antibodies could be easily scaled up, because they were derived from immortalized hybridoma cell lines. These antibodies could be used alone as unmodified antibodies to allow antibody-dependent cytotoxicity (ADCC) to occur. These antibodies could also be conjugated with radioisotope, chemotherapeutic drugs, or with cytokines or enzymes to target the cancer. Monoclonal antibodies are very unique to the tumor and it was thought they should only home towards the tumor and thereby limit toxicity to other cells. However, in retrospect, antibodies do not penetrate deeply within the tumor bed since they bind more heavily to the peripheral tumor cells that are initially exposed to the antibody.

The CC49 monoclonal antibody, which binds to a tumor-associated glycoprotein 72 (TAG-72), has also been tried in conjunction with radioisotopes [172]. Antibodies directed towards a cell surface ganglioside, called GD3, are being tried. GD3 is highly expressed in SCLC, but not in NSCLC. Bec2 is an anti-idiotypic antibody that binds to the idiotype of the antibody against GD3 and so it is thought to be a mimic of GD3. This antibody was combined with a BCG adjuvant to treat SCLC. These clinical studies against SCLC proved to be somewhat positive when compared to historic controls [173]. A similar approach was taken towards a Neu-glycosylated sialic acid ganglioside, called NeuGc-GM3, that is found on all types of LC. The anti-idiotypic antibody was called 1E10. This antibody was tried in both SCLC and NSCLC [174]. A survival benefit of about 6 months was

noted in those patients that developed immunity against NeuGc-GM3.

The first antibody (early 1990s) that seemed to have any major clinical effect against any type of cancer was the herceptin (Trastuzumab) antibody, which targets the her2/neu surface protein that was heavily overexpressed on some breast cancers [175]. At the time of its initial discovery, it was thought that this antibody would simply bind to a surface protein found on the cancer cell. Afterwards, it became apparent that the success was due to the fact that this antibody was interfering with a key cell-signaling pathway that prevented a growth factor signal pathway from being activated on the her2+ cells. The fact that this antibody also allowed antibody-dependent cell cytotoxicity (ADCC) probably helped its therapeutic efficacy [176]. So the take-home lesson is that it is vital to target a key biological factor that controls a unique aspect of the tumor and not just target any random cell surface tumor protein. Thus far, no antibody that directly targets an equivalent function of her2 on LC has been found. However, the creativity of medical scientists and the versatility of antibodies have allowed certain antibodies to be developed that enhance results to cancer to be developed and used in an indirect method (see Section 8).

7.3. Lymphokine-Activated Killer (LAK) Cells. Later immunotherapy studies focused on using a variety of other more advanced *ex vivo* cellular techniques, such as using lymphokine-activated killer (LAK) cells, cytotoxic T lymphocytes (CTLs) derived from tumor infiltrating lymphocytes (TIL) cells or from draining lymph nodes. The hope here was to generate overwhelming numbers of effector lymphocytes *in vitro* that could be applied *in vivo*. The genetic revolution of the 1980s and 1990s provided cancer immunologists with the opportunities to acquire sufficient amounts of cytokines and growth factors to stimulate the immune system on a large clinical scale. Some of the early cytokines that were genetically cloned and tested therapeutically were interleukin-2 (IL-2) and tumor necrosis factor (TNF). LAK cells became very popular in the mid-1980s. Human peripheral blood lymphocytes upon stimulation with IL-2 (a lymphokine known back then) turn NK and CD8+ T cells into nonspecific killer cells that lyse tumor cells *in vitro* [177]. When the cells were infused back into the patient, a clinical response was sometimes observed against melanomas and renal cancers [178]. One severe limitation of this therapy was that systemic toxicities occurred in many patients and this prevented many recipients from completing their therapies. This was perhaps due to the cytokines the LAK cells released such as TNF or IFN-γ. Enthusiasm for this modality also ran out when it became apparent that clinical responses were equally found when IL-2 was just administered *in vivo* alone without having to go through the laborious LAK cell collection and processing protocols [179]. Even though lung cancer cells were nonspecifically killed *in vitro*, LAK therapies with lung cancer patients largely failed. The review by Al-Moundhri and colleagues [180] covers the results of LAK therapy with lung cancers that was acquired from the 1980s.

Attempts were also made where advanced NSCLC patients were directly injected with IL-2 and TNF intratumorally [181]. This was a method of stimulating endogenous LAK cell precursors already within the tumor. Most patients experienced severe toxicities. Three patients had partial or stable disease that only lasted 6–9 months, but 1 patient with severe metastatic disease did live for at least 30 months [182].

7.4. Tumor Infiltrating Lymphocytes (TILs) and Draining Lymph Node (DLN) T Cells. The next great hope for immunotherapy involved isolating and expanding the tumor infiltrating lymphocytes (TILs). One early murine study fueled support for this concept [183]. This approach was novel because both CD4+ and CD8+ T cells could be isolated and expanded. The theory was that these lymphocytes should be specific for the tumor since they were already present within a tumor. This occurred in the days before the importance of antigen presentation by dendritic cells was known (see Section 7.6). It was observed that T cells needed to be "antigen-dosed" every 2 weeks or so, otherwise these T lymphocytes lost antigen specificity. There is a paucity of lymphocytes within many lung cancers [9]. So a derivative of this TIL approach was to take the lymphocytes from the tumor draining lymph nodes (DLNs) and expanding those cells *ex vivo* as you would do with the TIL. Both TIL and DLN cells were viewed to be very tumor specific and would have less toxicity than the LAK cells. Occasionally, these types of cells could have antitumor immune responses against cancers including lung [184, 185], but in most cases these cells did not work. With the subsequent discovery of Treg cells, the presence of these Tregs now explains why many of these TILs and DLNs expanded cells did not work as well as they were hoped because they were selectively enriched for Treg using the IL-2 (see Section 5.3.2(a)).

However, a big advance came about when it was discovered that if nonmyeloablative treatment was given to cancer patients before they received TIL cells, better clinical responses were seen [186]. Here other lymphocytes were killed, including Treg and other non-tumor-specific T cells. This mass killing of these lymphocytes then provided niches for these reinfused *ex vivo* expanded cells [187].

7.5. $\gamma\delta$ T Cells. Another T cell type also matures in the thymus, besides the classic $\alpha\beta$ T-cell receptor (TCR) rearranged T cells. These cells instead use a rearranged $\gamma\delta$ TCR to recognize their antigens. These $\gamma\delta$ T-cell receptors have a very restricted TCR diversity but are not MHC restricted. These cells may recognize nonclassic HLA-E and HLA-G molecules through NKG2D or Vγ9Vδ2 receptors. These lymphocytes were initially discovered to be cytotoxic towards leukemia cells. Wrobel and colleagues [188] discovered that these cells also had the ability to recognize and kill NCSLC lung cancer cells *in vitro*. Several of the ligands that $\gamma\delta$ T cells can recognize are MICA, MICB, ULBP-2 and ULBP-3 binding proteins found on the lung cancers [188]. Groh and colleagues [189] showed that *in situ* lung cancers possessed some ligands that $\gamma\delta$ T cells recognized; these $\gamma\delta$ cells were found *in situ* with the lung cancers. This non-MHC-restricted killing by $\gamma\delta$ T cells opens up

the possibility that allogeneic donors could be used for therapeutic purposes in lung cancers without risking the possibility of graft versus host reactions or autoimmune diseases. Clinical trials using $\gamma\delta$ T cells against recurrent NSCLCs are beginning to appear [190]. Ten patients were expanded with their autologous $\gamma\delta$ T cells, the median survival of these treated NSCLC patients was 401 days. Thus, this adoptive form of immunotherapy was deemed safe.

7.6. Gene Therapy. Gene therapy became the next big topic for a decade starting in the early/mid-1990s. The seminal work of Dranoff and colleagues [191] showed that immune-mediated rejection was not the same as generating long-lasting immunity. When living B16 melanoma cells were transduced with IL-2 or TNF and injected into mice, no tumor growth occurred as a result of CTL and NK becoming activated by those released cytokines. In contrast, IL-4- or GM-CSF-transduced cancer cells formed tumors when injected subcutaneously. However, if the IL-4 or GM-CSF transduced cells were irradiated and then used as a prophylactic vaccine, long-term memory was generated against the unmodified B16 tumor cells. Mice that rejected the IL-2- or TNF-transduced B16 melanoma cells showed no lasting recall memory, even though an immune response rejected the initial IL-2- or TNF-transduced tumor cells. Later it was realized that the released IL-4 and GM-CSF stimulated/recruited a poorly understood cell, which at the time was called the dendritic cell (DC). Today dendritic cells are considered the best antigen-presenting cells in the body. DCs can stimulate both naïve and previously activated T cells. This genetic engineering work produced a major paradigm shift that revolutionized our concepts in cancer immunology and has opened up the possibility of using immunotherapy against many different types of cancers.

Lung cancer vaccines transduced with various cytokines and costimulatory molecules have been used clinically. GM-CSF-transfected lung cancer cells used as vaccines are the most commonly used ones. Salgia et al. [192] was the first to use an autologous NSCLC tumor cells were transfected with an adenovirus that delivered GM-CSF. They used this in 97% of their patients. Here the tumor cells were isolated and transfected with adenoviruses. Two of the treated patients were noted to have been disease-free for 42+ months. A larger study was later run and was known as a GVAX approach [193]. The transduced cancer cells are irradiated and then used as a whole-cell vaccine. The longest surviving patients were noted to have received the most cells, which also had the highest expression of GM-CSF. A follow-up trial, called the allogeneic GVAX approach (allo-GVAX or Bystander GVAX) [194], was performed where allogeneic K-562 cells, which secreted much more GM-CSF than the autologous lung cancer cells, were combined with unmodified lung cancer cells. The results proved to be negative in terms of patient responses towards the cancers. One possibility is that this elevated dose of GM-CSF induced MDSC, which hindered antitumor immunity (see Section 5.3.2(d)). In conclusion, using autologous lung cancer cells that were transfected with the GM-CSF was the most beneficial vaccine.

Another genetic approach was to engineer lung cancer tumor cells with either HLA-A1 or HLA-A2 MHC molecules along with the immune costimulatory molecule, CD80. The idea here was to use a whole cell line (AD100) that expressed more MHC class 1 with the costimulatory molecules [195] to stimulate endogenous T cells directly by the vaccinated cells. Of their 18 patients tested, the median survival time was 18 months. No differences were noted in the responses of their patients to HLA compatibility, so this finding suggests that cross-presentation of tumor antigens was occurring, so HLA matching of the vaccinating tumor with the patient was not necessary to generate clinical responses.

A different genetic approach was taken with the canary pox virus. Here the virus genome was modified so that it would deliver a lung cancer antigen called the carcinoembryonic antigen (CEA) along with the B7.1 costimulatory molecule. This construct was called the ALVAC [196]. CEA is overexpressed in roughly 70% of NSCLC. This vaccine was injected intramuscularly every 4 weeks for 3 months into lung adenocarcinoma patients. No toxicities were seen with the highest doses of the virus given. Three patients had stable disease that correlated with CEA-specific T cells that produced IFN-γ. This project did not proceed any further with lung cancer but seems to be proceeding further with colon cancer.

CEA is used as a tumor vaccine with the common yeast, *Saccharomyces*, being used as the delivery vehicle. GlobeImmune (Louisville, Colorado) has pioneered this "Tarmogen" approach. Their clinical product is called GI-6207 and is used with metastatic adenocarcinomas. A phase 1 study enrolled 25 patients with three doses, which was administered at 4 sites subcutaneously biweekly for three months then monthly until disease progression. Twenty percent of the patients had stable disease and had declines in serum CEA levels [197].

The Mucin-1 (Muc-1) antigen is a core peptide of a glycoprotein found on many epithelial cancerous cells, including NSCLC. Muc-1 is thought to play several roles in cancer including loss of immune recognition, tumor cell migration, and resistance to apoptosis [198]. The attenuated Ankara strain of vaccinia virus was genetically engineered to transduce the Muc-1 antigen along with the IL-2 gene to create the "TG4010" vaccine. The vaccine was administered weekly by subcutaneous injections at the dose of 1.0×10^8 PFU and then once every 3 weeks until disease progression. There was an improved clinical outcome with TG4010 in patients, especially in those having T lymphocytes displaying an activated NK phenotype [199, 200]. The higher levels of activated T lymphocytes also correlated with longer TG4010 patient survival than the chemotherapy alone controls. In addition, increased circulating IFN-γ levels predicted a longer survival for the TG4010-treated patients.

7.7. Peptide Vaccine. A number of tumor-associated antigens have been discovered in lung cancers. Van der Bruggen et al. [201] have compiled a listing of various tumor antigens that have been found within human lung cancers. These tumor-associated antigens are composed of mutations, shared tumor-specific, differentiation, and overexpressed antigens.

These antigens could be possible antigens used for lung cancer immunotherapy.

A synthetic 25-amino-acid Muc-1 peptide was formulated into a liposome and is called L-BLP25 or Stimuvax [202]. This immunogen is now being used as a vaccine in NSCLC. The vaccine was injected into patients that received a single dose of cyclophosphamide. Sixteen of 65 patients demonstrated a T-cell immune response, and the patients had median survival time of 30.6 months compared to 13.3 months with the best supportive care.

The Wilms tumor antigen-1 (WT-1) is found within most NSCLCs and SCLCs [203]. Oka and colleagues [204] used a 9-mer of WT-1 (which is restricted for the HLA-A2402 allele) and emulsified it with the montanide ISA51 adjuvant. They administered this vaccine three times at 2-week intervals to breast, leukemia, and lung cancer patients who were HLA-A2402 positive and had WT-1-positive tumors. Three of the 10 lung cancer patients showed an immunological response as defined by a positive tetramer staining profile along with elevated intracellular IFN-γ expression. One patient has managed to survive the lung cancer and has been repeatedly vaccinated during this time (>2+ years).

Cyclophilin B was found on lung cancer adenocarcinomas and can be a target of CTLs [205]. Gohara and colleagues [206] used a cyclophilin-based peptide vaccine. Peptides were mixed with incomplete Fruends adjuvant (IFA) and injected as a subcutaneous vaccine in a phase I study in Japan. No significant increases in cellular responses were seen and this study was deemed to have failed.

The Mage-A3 peptide coupled with the AS02b adjuvant was tried in 182 patients that were Mage-A3+ NSCLC [207, 208]. This trial was using the GlaxoSmithKline MAGE-A3 protein. Some trends suggested beneficial results occurred and prompted further studies. These positive results initiated the development of the GSK1572932A study (ClinicalTrials.gov) and is part of the MAGRIT (MAGE-A3 Adjuvant Non-Small-Cell Lung Cancer Immunotherapy) study [209]. This study was opened in 2007 and is now closed to enrollment. This vaccine was composed of 13 intramuscular injections of the vaccine. Survival statistics are currently being collected to determine if these results are truly significant.

The IDM-2101 composite vaccine is made by IDM Pharma (Irvine, CA). This synthetic peptide vaccine is based upon 10 different HLA-A2-restricted epitopes against 5 different antigens (CEA, p53, Her2, Mage-2, and Mage-3 antigens along with a pan-DR epitope). A phase II study was done [210]. Survival was longer (17.3 months) in patients demonstrating an immune response to epitope peptides ($P < .001$) than those not immunologically responding. One patient had a complete response to the vaccine.

Epidermal growth factors (EGFs) are frequently overexpressed in LC and their receptors are frequently mutated within LC [211]. Hence, small chemical inhibitor strategies are targeted to the EGFR pathways and are frequently used in LC [212]. In this vaccination strategy, a recombinant fusion protein of EGF was conjugated to a bacterial P64K protein as a carrier protein to induce immune responses. As a result of this vaccination there was an increased titer of circulating anti-EGF antibody titers. This also correlated with a decreased level of serum EGF. They also made a direct correlation between antibody responses with patient survival, especially in those patients younger than 60 years old. This age response is very important, since as people age their immune responsiveness decreases. So vaccination may not be effective for individuals older than this age [213]. The data from 3 studies was complied in a meta-analysis and confirmed the study above [214].

7.8. Dendritic Cell Vaccines. Most smoking-related cancers have p53 mutations. DC-based vaccinations were based on infecting DC with p53 transfecting adenoviruses [215]. *In vitro*, when these transfected DCs are activated they can generate CTLs versus p53 [216]. These trials showed some progress. Introgen Therapeutics (Austin, Tx) in collaboration with the previous group is developing this concept with the INGN225 vaccine just with the p53 gene. In SCLC, this therapy induced a significant immune response and sensitized the SCLC to subsequent chemotherapy [217].

Hirschowitz and coworkers have developed an allogeneic 1650 adenocarcinoma cell line that was characterized for expression of Her2/neu, CEA, WT-1, Mage2, and survivin. They used these apoptotic cells to load immature autologous CD14+ monocytic DC stimulated with GM-CSF/IL-4. These DCs (80–90 million DCs) were injected as an intradermal vaccine to stimulate the immune system in a variety of stage IA to stage IIIB NSCLC patients [218]. Antigen-specific immune responses were noted in this study in the majority of the 16 patients tested. A follow-up study of these patients along with 14 new patients was reported 3 years later [219]. Many of these patients were still alive, although it was not clear whether these positive responses were due to good surgical resections or due to immunotherapy.

Dendritic cells are beginning to be developed as a therapy in China, which are pulsed with the Xage-1b protein [220]. Xage-1b is a member of the cancer-testis family of antigens and is overexpressed in many lung cancers. In early studies, this methodology does generate CTLs *in vitro* and has the ability to kill lung cancers, but not normal lung cells. Thus, this antigen might be added to the tumor antigen armaments towards lung cancer.

Tumor lysates derived from autologous NSCLCs are being electroporated into dendritic cells in Korea. These DCs were then injected into advanced NSCLC patients. In these early studies, Um and coworkers [221] showed that when their patients received the most dendritic cells (12 million cells) 3 times at 2-week intervals, five out of the nine patients resulted in increased IFN-γ production after an *in vitro* restimulation. Two of the patients treated with these cells appeared to have some beneficial effects. So getting tumor antigens into dendritic cells can be effectively done in a couple of ways.

When people view tissue culture cells under the microscope, one can frequently see remnants of cells, left as a cell moves away. These released cell-debris particles are called exosomes. These exosomes contain all the small material as the cell that produced them, proteins, RNA, microRNA, and so forth, including tumor antigens. Some

of these exosomes when formulated with CpG adjuvant and injected into animals can create immune responses towards the original tumor [222]. This intriguing observation was followed up when exosomes from mature dendritic cells were used as the vaccine [223–225]. This work has now been developed into a clinical modality for treating NSCLC at the Institut Gustave Roussy in France. Drs. Besse and Chaput are spearheading this approach. In this trial autologous DCs are being loaded with HLA-DP04-restricted MAGE-3, and HLA-A02-restricted peptides NY-ESO-1, MAGE-1, MAGE-3 and MART-1. So far, no results of this clinical effort have been reported.

7.9. Knockout Strategies. The advantage of using a whole cancer cell is that the entire spectrum of tumor antigens can be harnessed against the tumor; all these antigens can now stimulate multiple clones of T cells. This contrasts with vaccination strategies that only use a few antigens, such as the peptide vaccines or adenoviruses transfecting tumor antigens. For immune responses to occur, APC must take up the antigen and chop it up via the DC proteosome. These digested peptides should then be presented via the patient's own DC and stimulate the host T cells. In theory, any tumor cell could be used to vaccinate the patient, regardless of the patient's HLA profile, as the Raez et al. study [195] seems to show. NovaRx (San Diego, CA) has genetically knocked down TGF-β_2 expression and is using a combination of 4 allogeneic cell lines (lucanix also known as belagenpumatucel-L) as a vaccine [226]. Since TGF-β is prevented from being made, when these killed tumor cells are taken up by the DCs they should respond maximally, since no endogenous TGF-β was present to hinder DC function. Hence, better immune stimulation should occur. Early results in a phase II study do suggest the vaccine is well tolerated. It also generated T cell reactivity in 11 of 13 of these treated lung cancer patients. As a result, survival of these immune responding patients was 32.5 months compared to 11.6 months with the nonresponders. Thus, allogeneic cells do seem to show they can act as an effective immunogen, although it is still really too early to make it an established fact.

8. Antibodies That Can Augment Immune Responses

In contrast to antibodies being used to directly treat the cancer, antibodies can create positive clinical responses in other ways. One strategy of antibodies is to either use them as an antiangiogenic approach or use antibodies (anti-CTLA-4, anti-PD-1/PD-L1, and anti-IL-2 receptors) to inhibit some of the negative regulatory pathways. The former antibody therapy is beginning to show clinical responses in many cancers, including NSCLC, while the later antibodies may be added to other therapies as discussed earlier. These combined therapies could have a potentially big impact on lung cancer immunotherapy. So far, none of these approaches has been reported with lung cancer. But we anticipate we will see more of these types of studies in the near future.

8.1. Bevacizumab Is an Anti-VEGF Antibody Developed by Genentech. VEGF, as described in Section 5.3.1(c), cannot only play a role in tumor angiogenesis by working on the tumor recruitment of endothelial cells but may also play a role in breaking autocrine loops in LC. VEGF can inhibit immune responses by turning off the actions of dendritic cells. Bevacizumab has recently been approved by the US FDA as a first-line therapy for metastatic colorectal cancer. And it also does appear to be effective against other human cancers, too [227]. In a phase 3 study done with metastatic NSCLC patients, there was increased progression-free survival ($P < 0.0001$). Additionally, there was a better overall survival (12.5 months compared to 10.2 months) with a $P < 0.007$. So some progress is now being made against lung cancer using this antibody.

8.2. Ipilimumab Is the Antibody That Targets an Immunomodulatory Molecule Called CTLA-4. As T cells become activated into effector cells, they are induced to express CTLA-4 antigen, which is capable of binding to the costimulatory molecules found on DC, CD80, and CD86. CTLA-4 binds better to the CD80/CD86 molecules than does the immunostimulatory CD28 receptor found on the T cells [118]. Negative signals are delivered to the T cells upon binding to APC CD80/CD86 molecules via CTLA-4 and the T cell is essentially inhibited from further functional activities. These T cells will eventually be eliminated via apoptosis. Both natural and induced Tregs also use CTLA-4 (cytotoxic T lymphocyte antigen-4) to inhibit immune responses by inhibiting APC function. Antibodies towards CTLA-4 such as ipilimumab are being developed to inhibit this pathway to improve tumor vaccines in humans. This antibody is now being used in NSCLC [228]. By preventing this CTLA-4-mediated downregulation, an enhanced immune response can be made and can probably enhance antitumor immune responses. Recently, this antibody has been successfully used for the treatment of melanoma [229]. Here, additional, four months of survival were noted in these patients. In general, these antibodies have to be carefully watched since they have the potential to cause autoimmunity and produce other severe effects that limit their therapeutic ability.

8.3. Another Family of Antibodies Is Directed towards the Family of PD-1/PD-L1 Inhibitory Molecules [230]. The PD-1/PD-L1 system can also be considered a tumor counterattack strategy. The programmed death-1 (PD-1) molecule is a member of the CD28 family. The ligands for PD-1 (PD-Ls) are PD-L1 (B7-H1) and PD-L2 (B7-DC). Upon signal transduction with these proteins the T cells become triggered for cell death via apoptosis. PD-L1 has been detected on human lung cancers. Dong et al. [231] showed 95% of the lung cancers (20 of 21, including adenocarcinomas, squamous and small cell) were positive for B7-H1(PD-L1). Iwai and colleagues [232] also showed a similar type of mechanism. Brown et al. [233] also showed 6 of 6 adenocarcinomas and 8 of 8 squamous cancers were strongly positive for PD-L1(B7-H1). When *in situ* lung cancers expressed more B7-H1, there were fewer T cells present than in the B7-H1 negative cancers [234]. Perrot et al. [235]

also showed that the myeloid dendritic cells that infiltrated NSCLC were blocked in an immature state and were B7-H1+.

Besides the B7-H1 and B7-DC molecules, a couple of other family members with similar biological functions, namely B7-H3 and B7-H4, are expressed on various NSCL cell lines. These markers are found within *in situ* squamous and large cell carcinomas [236, 237]. Roughly half of lung cancers expressed at least one of these markers.

A variety of antibodies from various companies, Bristol-Myers Squibb, Merck, GlaxoSmithKline, and Cure Tech, are targeting these receptors/ligands, which also can inhibit T cell and NK cell responses. One interesting fact is that these antibodies do not have as much toxicity as the anti-CTLA-4 antibodies seem to possess [124]. Thus, this antibody will probably be the best way to target the immunosupressor cells.

8.4. The Previous Use of an ONTAK Immunotoxin Which Depletes Treg [238] via the High-Affinity IL-2 Receptor (See Section 5.3.2(a)) Is Another Way to Target This Same Receptor. Daclizumab [239] targets the high-affinity interleukin-2 receptor-α on Treg cells. By eliminating Treg cells, a more sustained antitumor immune response can also be maintained. This therapy is currently being tried in melanoma patients at the University of Chicago and in glioma patients at Duke University. Here the idea is to eliminate the Treg before they inhibit the optimal anti-cancer immune response. Since LC has high concentrations of Treg, this could prove to be very good at improving clinical results.

9. Summary

Education is often considered a painful process, and that is certainly true in the case of lung cancer. Each new lesson requires expensive clinical trials to learn this vital information. Each cancer has its own unique set of tricks to avoid clinical therapies. Lung cancers use a variety of defensive strategies to escape chemo- and radiation therapies also can serve double duty by resisting the immune system. Immunologists are beginning to design rational therapies to counteract these defensive strategies. It will still take work to develop effective therapies against this killer cancer. Most likely, no single immunotherapy will work as a stand-alone therapy and it will have to be combined with other therapies to achieve a cure. Progress with immunotherapy is slowly being made against other cancers previously considered terminal cancers, that is, melanoma, glioma, and castrate-resistant prostate cancer. The lessons learned in those clinical trials can certainly be applied towards lung cancer.

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Review Article

New Roads Open Up for Implementing Immunotherapy in Mesothelioma

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Treatment options for malignant mesothelioma are limited, and the results with conventional therapies have been rather disappointing to this date. Chemotherapy is the only evidence-based treatment for mesothelioma patients in good clinical condition, with an increase in median survival of only 2 months. Therefore, there is urgent need for a different approach to battle this malignancy. As chronic inflammation precedes mesothelioma, the immune system plays a key role in the initiation of this type of tumour. Also, many immunological cell types can be found within the tumour at different stages of the disease. However, mesothelioma cells can evade the surveillance capacity of the immune system. They build a protective tumour microenvironment to harness themselves against the immune system's attacks, in which they even abuse immune cells to act against the antitumour immune response. In our opinion, modulating the immune system simultaneously with the targeting of mesothelioma tumour cells might prove to be a superior treatment. However, this strategy is challenging since the tumour microenvironment possesses numerous forms of defence strategies. In this paper, we will discuss the interplay between immunological cells that can either inhibit or stimulate tumour growth and the challenges associated with immunotherapy. We will provide possible strategies and discuss opportunities to overcome these problems.

1. Introduction

Links between cancer and inflammation were first noted by Rudolf Virchow in 1863, on observations that tumours often arose at sites of chronic inflammation and that inflammatory cells were present in biopsy samples from tumours [1]. In a severe combined immunodeficiency (SCID) mouse xenograft model, it has recently been shown that inflammation precedes the development of human malignant mesotheliomas [2]. Also, epidemiological studies have revealed that chronic inflammation caused by chemical and physical agents, autoimmune and by inflammatory reactions of uncertain aetiology, predisposes for certain forms of cancer [3, 4]. Recently our group demonstrated a significantly shorter survival in patients with lung cancer in subjects with a history of pulmonary tuberculosis than patients without tuberculosis [5], revealing even a more complex interplay between inflammation and cancer. Increasing evidence indicates that the inflammation-cancer connection is not only restricted to the initiation of the cancer process, since all types of clinically manifested cancers appear to have an active inflammatory component in their microenvironment. These experimental findings and clinical observations have led to cancer-related inflammation being acknowledged as an important hallmark of cancer [6].

2. Immunooncology

2.1. Tumour-Immune Surveillance. Old, Klein, and others investigated murine tumour transplantation models and showed that the immune system of healthy recipient mice

was able to distinguish transformed malignant cells from normal cells [7, 8]. Even preceding these publications, Frank MacFarlane Burnet and Lewis Thomas formulated their cancer immunosurveillance hypothesis: "It is by no means inconceivable that small accumulations of tumour cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumour and no clinical hint of its existence" [9]. At that time this hypothesis was controversial; however, with the current knowledge and ongoing research, it is apparent their premise seems to be correct because there is strong evidence from animal studies that cells of the immune system carry out surveillance and can eliminate nascent tumours [10].

Tumour-associated antigens (TAAs) are antigens acquired by tumour cells in the process of neoplastic transformation that can elicit a specific immune response by the host. It is known that several immunological cell types are involved in the recognition and destruction of tumours during early stages of development. These include cells and factors of the innate immune system, including macrophages, neutrophils, complement components, $\gamma\delta$ T cells, natural killer (NK) cells, NKT cells and certain cytokines (IL-12, IFN-γ) and cells of the adaptive immune system, including B lymphocytes, helper T cells (Th cells), and cytotoxic T lymphocytes (CTLs).

TAAs need to be presented to the cells of the adaptive immune system. Dendritic cells (DCs) are widely acknowledged for their potent antigen presenting capacity and play a key role in the initiation of this adaptive immune response by activation and modulation of lymphocyte subsets [11]. DCs originate from bone marrow precursor cells and are found at low frequencies in peripheral tissues where they maintain an immature phenotype and search their surroundings for foreign substances. Immunogenic TAAs are secreted or shed by tumour cells or released when tumour cells die and can be taken up by DCs or other antigen presenting cells (APCs). Upon encountering an antigen, DCs mature and migrate to regional draining lymphoid organs. The captured antigen is processed and presented by major histocompatibility complex (MHC) class I and class II molecules on their cell membrane leading to the activation of antigen-specific lymphocytes. This results in antibody production by B lymphocytes and tumour-specific CTLs to assist the innate immune responses in the killing of tumour cells.

2.2. Tumour-Immune Escape. Increasing evidence reveals that when tumours progress in time, tumour cells undergo changes to escape immune surveillance. This process encompasses three phases: elimination, equilibrium, and escape. During the first phase, tumour cells have to escape the immune surveillance to survive. Then these surviving tumour cells can enter the equilibrium state, in which there is equilibrium between tumour growth and tumour killing by cells of the immune system. In this stage, tumours can persist for years without progressing to more severe tumour stages. However, during this period, tumour cells may undergo mutations caused by their genetic instability, potentially generating variants that can escape the immune system, by either evading the induction of an immune response or by inhibiting antitumour responses via a variety of immune suppressive mechanisms.

2.3. Immune Suppressive Mechanisms. The tumour immune escape mechanism can be greatly enhanced by the induction of an immune suppressive tumour microenvironment. In this microenvironment, inflammatory cells and molecules have a major influence on cancer progress. Effective adaptive immune responses are suppressed through the activation of several pathways. For example, the differentiation and activation of dendritic cells, which are the key initiators of adaptive immune responses, are inhibited by signals (such as IL-10 and VEGF) present in the tumour microenvironment. In addition, tumours, peripheral blood, and lymph nodes contain increased amounts of regulatory T cells (Tregs), which suppress both the adaptive and innate immune responses [12]. Also, a heterogeneous population of myeloid-derived suppressor cells (MDSCs) are induced in tumour-bearing hosts; these cells, as well as tumour-associated macrophages (TAMs or M2 macrophages), are potent suppressors of antitumour immunity. Not only do MDSCs and TAMs suppress the antitumour response, but they also assist the malignant behaviour of tumour cells by secreting cytokines, growth factors, matrix-degrading enzymes, and proteases, which promote tumour progression or enhance metastasis.

In conclusion, immune cells can either protect the host against cancer development or promote the emergence of tumours with reduced immunogenicity leading to a complex interplay of tumour growth and tumour regression mechanisms (Figure 1) [13].

3. Immunotherapy

Cancer immunotherapy attempts to activate or enhance the antitumour effects of the immune system of the patient, or it may assist in the capabilities of the immune system to fight cancer. Multiple approaches for immunotherapy have been developed over the years, and many are in various stages of (pre-)clinical research. Immunotherapy can be divided into two main categories: passive and active immunotherapy [14].

3.1. Passive Immunotherapy. Passive immunotherapy makes use of *in vitro* produced immunologic effectors that are capable of influencing tumour cell growth. The most common form of passive immunotherapy is called monoclonal antibody therapy. It consists of humanized monoclonal antibodies that are investigated in several human malignancies. Monoclonal antibodies can target cells directly [15] or indirectly. Monoclonal antibodies are also used as immune modulators to inhibit immune suppressive molecules/cells or activate immune stimulatory molecules. Efficacy of this approach can sometimes be enhanced by linking a toxin to these antibodies (e.g., radionucleotides or anticancer drugs).

In mesothelioma, preclinical studies targeting mesothelin with immunotoxins CAT-5001 (formerly SS1P) and amatuximab (previously known as MORab-009) were

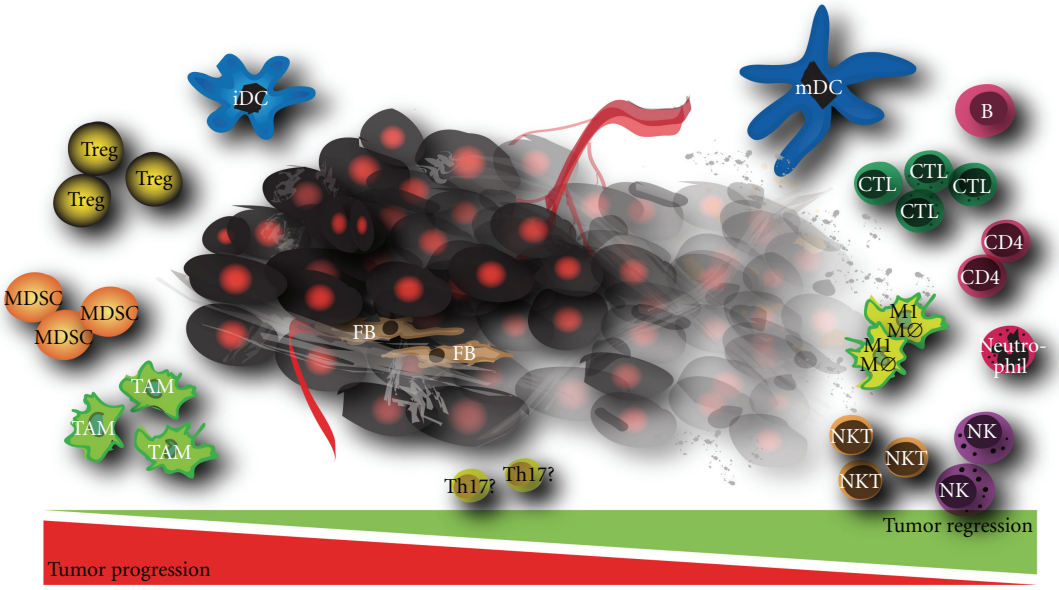

Figure 1: Interplay between immunological cells that inhibit tumour growth on the right of the tumour and cells that aid in tumour progression on the left. (Tumour is depicted as black cells with a red nucleus in the middle.) iDC: immature dendritic cell, Treg: regulatory T cell, MDSC: myeloid-derived suppressor cell, TAM: tumour-associated macrophage, mDC: mature dendritic cell, B: B cell lymphocyte, CTL: cytotoxic T lymphocyte, M1 MØ: M1 macrophage, NK(T): natural killer (T) cell, Th17: helper T lymphocyte 17, FB: fibroblast.

promising [16–18] and therefore progressed to clinical trials. CAT-5001, administered to mesothelioma patients, among other cancer types, showed only modest clinical responses [17, 18]. Amatuximab failed to demonstrate any radiological responses in a phase I trial in mesothelioma and other cancer types [19]; however preclinical studies demonstrated significant antitumour efficacy using combination of amatuximab and chemotherapy treatment [20] justifying a multicenter phase II clinical trial utilizing cisplatin/pemetrexed with amatuximab in mesothelioma patients. This trial has been completed and results are expected soon. More recently a phase I study of SS1(dsFv)PE38, a recombinant antimesothelin immunotoxin, was commenced which is ongoing at this moment (ClinicalTrials.gov Identifier: NCT00575770).

Another method of passive immunotherapy uses adaptive transfer of (autologous or allogeneic) antigen-specific effector cells (like T cells and NK cells) that can be expanded and/or activated *ex vivo* and subsequently administered to the patient to attack the tumour [21]. This approach showed the potential to reconstitute host immunity against pathogens, like Epstein-Barr virus (EBV) in immune suppressed patients, but more importantly also provides evidence that adaptive T cell transfers can prevent the induction of EBV-associated lymphomas [22]. This led to the concept that antigen-specific T cell transfer can be used as an antitumour therapy to eradicate established tumours. The approach of adaptive T cell transfer to eradicate malignancies is challenging [23].

3.2. Active Immunotherapy. Active immunotherapeutic approaches aim at inducing or boosting immune effector cells *in vivo* against tumour cells, through the administration of immune mediators capable of activating the immune system.

Several cytokines are capable of activating and recruiting specific immune cells that can enhance antitumour immunity (e.g., IL-2, IL-12, IL-15, TNF-α, GM-CSF). These cytokines can be used as single agent or in combination with other immunotherapeutic strategies.

Defined TAA epitopes have been used to vaccinate cancer patients [24]; however this approach is limited by the relatively low number of identified specific peptides and by the requirement of MHC typing. By using the whole TAA protein for immunization, the need of peptide identification can be circumvented. These proteins can be taken up by APCs and endogenously processed into epitopes for presentation to T cells. Adjuvants need to be added to induce APCs activation and avoid tolerance induction [25].

DNA sequences coding for specific TAAs can be directly injected into the skin. DNA then needs to be taken up, transcribed into mRNA, translated into a protein, and processed into peptides by APCs.

In mesothelioma, the TAA's mesothelin and Wilms tumour-1 (WT-1) are highly expressed and thought to be physiologically relevant to this tumour type [26]. In the Memorial Sloan-Kettering Cancer Center a phase I peptide vaccination clinical trial in mesothelioma patients is ongoing (ClinicalTrials.gov Identifier: NCT01265433). In these patients, inoculation with WT-1 peptide elicited WT-1-specific CD4 and CD8 T-cell responses, with minimal toxicity [26]. TroVax has been shown to stimulate an immune response to a particular protein widely found on mesothelioma cells called 5T4; a clinical trial testing the effectiveness of TroVax is currently active in the Wales Cancer Trials Unit (ClinicalTrials.gov Identifier: NCT01569919).

An important restriction of this method is the relatively inefficient delivery into APCs. Viruses engineered to express TAAs can be injected directly into the patient. The virus then infects the host cell, leading to cell death and presentation of antigenic epitopes to the immune system. A wide variety of viral vectors are available. Currently, a trial using intrapleural administration of a vaccine with a measles virus strain is performed at the Mayo Clinic (ClinicalTrials.gov Identifier: NCT01503177). However there are concerns regarding the immune dominance of viral antigens over TAAs, resulting in a strong anti-virus response leading to virus eradication and attenuation of the antitumour immune response [27].

DCs have emerged as the most powerful initiators of immune responses. In the natural activation of the adaptive immune system against tumour cells, DCs play a crucial role since they are capable to engulf tumour antigens and activate lymphocytes in an antigen-specific manner. Therefore, the application of DCs to therapeutic cancer vaccines has been prompted [28].

The research group of Dr. Robinson published a very interesting trial, in which they used an autologous tumour lysate vaccine that was manufactured from surgically resected mesothelioma material and administered subcutaneously together with granulocyte-macrophage colony stimulating factor (GM-CSF). GM-CSF facilitates APCs recruitment and survival *in vivo* which in turn may generate tumour-specific immunity after uptake of the TAA from the lysate. Twenty-two patients were enrolled onto this trial. Of these, five developed positive delayed type hypersensitivity skin tests, and five showed evidence of altered antibody specificities by western blotting, proving that GM-CSF could induce tumour-specific immunity, both cellular and humoral responses. 32% of the patients developed at least one type of anti-MM immune response. Furthermore, the therapy was safe and was associated with stable disease; however no major tumour regressions were observed [29].

While this study showed potential for GM-CSF as immunotherapeutical approach, *in vivo* stimulation of APCs is also a very attractive method. Sipuleucel-T is an active cellular immunotherapy consisting of autologous peripheral-blood mononuclear cells (PBMCs), including APCs. Recently, Kantoff et al. published a phase III trail where they used *ex vivo* activated Sipuleucel-T with a recombinant fusion protein (PA2024). PA2024 consists of a prostate antigen, prostatic acid phosphatase that is fused to GM-CSF, an immune-cell activator. Sipuleucel-T prolonged survival among men with asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer [30], providing evidence for cell-based immunotherapeutic agents in solid tumours.

In mesothelioma, the source of the TAA for DC loading remains a critical issue that will determine the efficacy of the DC-based vaccination. A careful identification and characterization of antigenic epitopes is needed when peptides want to be used. However, the ideal source of TAAs may be the tumour itself, since it expresses all the TAAs that can be targeted.

Incubating DCs with dead tumour cells (necrotic or apoptotic cells), as was shown in a pioneering article by the research group of Dr. Gregoire, DCs were exposed to a full array of antigenic peptides that rapidly gain access to both MHC Class I (cross-presentation) and MHC Class II pathways, therefore leading to a diversified immune response involving cytotoxic T lymphocytes (CTLs) as well as CD4+ T cells. In their paper they successfully demonstrated *in vitro* culture and antigen loading in a human mesothelioma model, resulting in a specific CTL response [31].

One of the advantages of an *ex vivo* culture model is that DCs can be generated in large amounts and pulsed with tumour antigens under optimal conditions. In mesothelioma, we previously investigated the effect of DC-based immunotherapy on the outgrowth of mesothelioma in a murine model [32]. We established that DC-based immunotherapy induced strong tumour-specific CTLs responses leading to prolonged survival in mice. The efficacy of immunotherapy was dependent on the tumour load; most beneficial effects were established at early stages of tumour development.

On the basis of these preclinical animal studies, we have performed the first clinical trial in which autologous tumour lysate-pulsed DCs were administrated in mesothelioma patients [33]. Patients were eligible for the study when sufficient tumour cells could be obtained from pleural effusion or tumour biopsy material at the time of diagnosis. DC-immunotherapy was planned after completion of the cytoreductive therapy provided that during chemotherapy no major side effects occurred and there was no progressive disease. Patients received three immunizations with mature DCs, loaded with autologous tumour lysate. Each immunization, consisting of $50 \times 10e6$ cells, was administered intradermally and intravenously (Figure 2). Overall, the vaccination regimen with loaded DCs was well tolerated, and a successful immune reaction was induced by the DC vaccinations.

The University Hospital of Antwerp has started a similar protocol in mesothelioma and several other solid tumours but is using WT-1 as antigen loading for the DCs (ClinicalTrials.gov Identifier: NCT01291420), circumventing the need for patient's tumour material.

Another method to load DCs is to make use of measles-virus-infected mesothelioma cells. It was shown that this method induced spontaneous DC maturation and that priming of autologous T cells by DCs loaded with measles-virus-infected mesothelioma cells led to a significant proliferation of tumor-specific CD8 T cells [34].

4. Improving Immunotherapy

While immunotherapy was proven safe and feasible, it has not established its place yet in mesothelioma treatment. Partly, this is due to the presence of immunosuppressive cells in peripheral blood, lymphoid organs, and within the tumour environment that hamper immunotherapeutic treatments. Several strategies have been performed or are currently tested that target the immunosuppressive cells aiming to improve the efficacy of immunotherapy. In the following sections, we will focus on three populations of

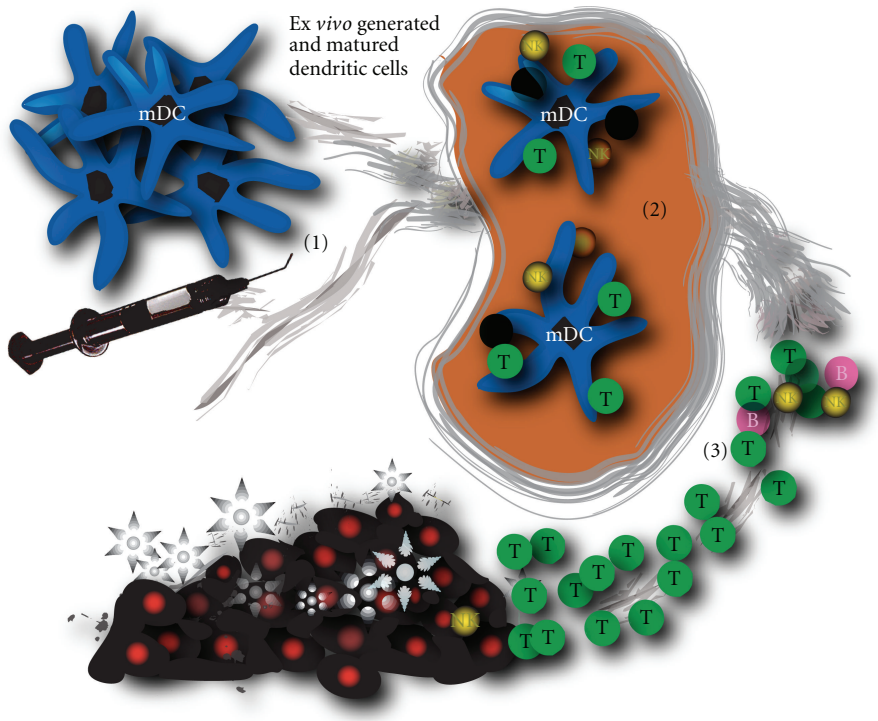

Figure 2: Schematic drawing showing the administration of *ex vivo* cultured mature dendritic cells into a patient (1), resulting in antigen presentation in the lymph node (2) and a specific antitumour cytotoxic antitumour response (3). Tumour cells are depicted as dark cells.

suppressive cells, the MDSCs, Tregs, and TAMs, that are increased in most cancer patients. It is becoming increasingly clear that these populations contribute to the impaired antitumour responses frequently observed in cancer patients. Therefore, combating immunosuppression through modulation of these cell types will be an important key to increase the efficacy of immunotherapy and should lead to better prognosis for cancer patients.

4.1. Myeloid-Derived Suppressor Cells. MDSCs are a heterogeneous population of bone-marrow-derived myeloid cells, comprising of immature monocytes/macrophages, granulocytes, and DCs at different stages of differentiation [35]. A subset of MDSCs, mononuclear MDSCs (MO-MDSCs), is mainly found at the tumour site while polymorph nuclear MDSCs (PMN-MDSCs) subset is found in blood, lymphoid organs, and at the tumour site. They express a number of surface markers, that are on themselves not unique but in combination can define MDSCs. MDSCs are increased in cancer patients, and it is anticipated that they play a suppressive role during the innate and adaptive immune responses to cancer but have also been described in the course of other pathologic processes such as thermal injury, various infectious diseases, sepsis, trauma, after bone marrow transplantation, and in some autoimmune disorders.

Activation of MDSCs not only requires tumour-derived factors (e.g., tumour-derived prostaglandin E2 (PGE2)) but also IFN-γ produced by T cells and factors secreted by tumour stromal cells (like IL-1β, IL-4, IL-6, IL-10, IL-13). Activation of cytokine receptors on MDSCs leads to activation of STAT-signalling pathways, resulting in the production of immune suppressive substances (like TGF-β, reactive oxygen species (ROS), and nitric oxide synthetase (NOS)).

MDSCs can inhibit the antitumour immune response in several ways.

(i) MDSCs are capable of producing reactive oxygen species (ROS) and peroxynitrite, which is responsible for most of the adverse effects on T cells, linked to ROS. Changes caused by nitration of the T cell receptor make T cells incapable of interacting with the MHC complex on APCs, which is necessary to obtain T cell-specific stimulation [36, 37].

(ii) MDSCs can inhibit the antitumour response in an antigen nonspecific manner by the high expression of the enzyme inducible nitric oxide synthetase (iNOS), leading to the generation of NO. NO can suppress T cell function though various mechanisms including the inhibition of the cell signalling pathways and inducing DNA damage to T cells.

(iii) Arginase-I activity by MDSCs depletes L-arginine from the environment, contributing to the induction of T cell tolerance by downregulating the CD3ζ-chain expression of the T cell receptor [38, 39].

(iv) MDSCs block T-cell activation by sequestering cysteine and thus limiting the availability of the essential amino acid cysteine [40].

(v) MDSCs can inhibit T cell proliferation by producing IL-10 and TGF-β [41].

(vi) Antitumour cells, like NK- and NKT-cells, can be inhibited by MDSCs via TGF-β1-dependent mechanisms. MDSC can bind to the TGF-β receptor on target cells via membrane-bound TGF-β, leading to activation of intracellular pathways resulting in downregulation of NK-specific receptors [41].

(vii) The plasma membrane expression of enzyme ADAM17 on MDSCs cleaves L-selectin on naïve T cells, decreasing their ability to home to sites where they could be activated [42].

(viii) MDSCs can indirectly enhance immune suppression via the induction of Tregs [43–45].

(ix) MDSCs differentiate under certain biological conditions into mature functionally competent macrophages or to DCs influencing tumoural responses [46].

4.2. Targeting MDSCs. Both gemcitabine and 5-fluorouracil (5FU) have shown to be selectively cytotoxic on MDSC in murine tumour models [47]. The treatment of tumour-bearing mice with 5FU led to a decrease in the number of MDSC in the spleens and tumour beds of animals whereas no significant effect on T cells, NK cells, DCs, or B cells was noted. 5FU showed a superior efficacy over gemcitabine to deplete MDSC and selectively induced MDSC apoptotic cell death [47].

Gene expression profile analysis of multiple tumour types identified SCF (c-kit ligand) as a candidate tumour factor involved in MDSC accumulation. Inhibiting c-kit using the tyrosine kinase inhibitor sunitinib resulted in a decrease of the number of MDSC and Treg in advanced tumour-bearing animals [49].

The production of ROS by MDSCs, which is responsible for most of the adverse effects on T cells, is highly depending upon cyclooxygenase-2 (COX-2) enzyme activity [50]. The inducible COX-2 enzyme is essential in the biosynthesis of prostaglandins. Celecoxib is a selective COX-2 inhibitor. Therefore, we investigated the effect of celecoxib treatment on the four MDSC subsets that were identified in the spleen of tumour-bearing mice [51]. When combining DC-based immunotherapy and celecoxib treatment, a significant improvement of the immunotherapy was seen in comparison to no or single modality treatment. Treatment of tumour-bearing mice with dietary celecoxib prevented the local and systemic expansion of all MDSC subtypes, and also their suppressive function was impaired. At the National Cancer Institute, allogeneic tumour cell vaccine is combined with celecoxib and metronomic oral cyclophosphamide as adjuvants in thoracic malignancies (ClinicalTrials.gov Identifier: NCT01143545); the rationale for using cyclophosphamide is discussed further in this paper.

4.3. Tumour-Associated Macrophages. Macrophages are a major component of the leukocyte infiltrate in the tumour microenvironment [52] and have even been described as key orchestrators of cancer-related inflammation [53]. Classically activated (M1) macrophages, following exposure to IFN-γ, have antitumour activity and tissue-destructive activity. In response to IL-4 or IL-13, macrophages undergo alternative (M2) activation. M2 macrophages are oriented to tissue repair, tissue remodelling, and immune regulation. TAMs generally have the phenotype and functions similar to M2 macrophages and display a defective NF-κB activation in response to different proinflammatory signals [54].

TAM recruitment in tumours is mediated by several cytokines, of which CCL2 seems to be the main player; other chemokines involved in monocyte recruitment are CCL5, CCL7, CXCL8, and CXCL12, as well as cytokines such as VEGF, PDGF, and the growth factor M-CSF [53]. It has been shown that MO-MDSCs are capable of differentiating towards TAMs. Therefore, similar recruitment factors are described that contribute to the infiltration of TAMs and MDSCs into tumour tissue [55]. In addition, dynamic changes of the tumour microenvironment occur during the transition from early neoplastic events toward advanced tumour stages resulting in local hypoxia, low glucose level, and low pH. These events in the tumour microphysiology drive the switch from a M1 macrophage toward the M2 type.

TAMs are able to suppress the adoptive immune response through various mechanisms and contribute to angiogenesis and tumour invasiveness.

(i) TAMs are able to produce immune suppressive cytokines, like CCL17, CCL18, CCL22, IL-1β, IL-6, IL-10, and TGF-β. IL-10 in combination with IL-6 can lead to upregulation of molecules in TAMs, which are implicated in suppression of tumour-specific T cell immunity [56].

(ii) TAMs express the enzyme indoleamine 2,3-dioxygenase (IDO), a well-known suppressor of T cell activation. IDO catalyzes the catabolism of tryptophan, an essential amino acid acquired for T cell activation [57].

(iii) TAMs contribute to immune suppression via indirect ways. Secretion of CCL18 leads to recruitment of native T cells. Attraction of naive T cells into the tumour microenvironment is likely to induce T cell anergy [58]. Besides CCL18, CCL17 and CCL22 are abundantly expressed. These cytokines interact with CCR4 receptor expressed by Tregs and induce T-helper 2 polarization [59]. Via expression of VEGF, TAMs can block antigen uptake by APCs and attract MDSCs, which can function as TAM precursors but are also actively suppressing T cell function. MDSCs are depending on prostaglandin E2 (PGE2) for their function. PGE2 is secreted by many types of cancer; however, TAMs are also capable of producing PGE2 and therefore assist MDSC function [60].

(iv) In tumour stroma, TAMs produce matrix metalloproteases (MMP) and other proteases, leading to degradation of the extracellular matrix. During this process, several cytokines, chemokines, and growth factors are released from the matrix that promotes

and facilitates endothelial cell survival and migration and thereby enhances angiogenesis [61].

(v) Besides indirect mechanisms, angiogenesis is also directly stimulated by TAMs. TAMs can produce proangiogenic factors like vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, and platelet-derived growth factors (PDGFs). The release of these factors leads to the neovascularisation, especially in hypoxic regions within the tumour [53, 62].

(vi) In addition to angiogenesis, TAMs are also strongly involved in lymphangiogenesis, a process mediated by a number of factors including VEGF-C and VEGF-D via VEGFR3 [53].

(vii) Outside the scope of the tumour microenvironment, but a pivotal step in general tumour biology, TAMs cooperate on tumour dissemination by promoting invasion characteristics. One of the main factors involved significantly is TNF-β: coculture of neoplastic cells with macrophages enhances invasiveness of malignant cells through TNF-dependent MMP induction by macrophages [53].

4.4. Targeting TAMs. There is accumulating evidence supporting the hypothesis that effects on TAMs may contribute to the antitumour effect of bisphosphonates [63]. We investigated the effect of zoledronic acid (ZA) in mesothelioma-inoculated mice. Our data showed that the addition of ZA to macrophage-inducing culture conditions significantly inhibits the upregulation of F4/80, MHCII, and CD11c. In addition, these data reveal that adding tumour supernatant leads to polarization of the macrophage phenotype towards M2 subtype and that ZA can prevent this polarization *in vitro*, leading to a significant reduction in the CD206 expression on macrophages cultured in the presence of ZA. *In vivo*, however, no significant differences on tumour progression and survival could be observed between untreated mice and mice treated with ZA, because the reduction in TAMs was associated with an increase in MDSC [64].

IL-6 stimulates tumour macrophage infiltration in ovarian cancer, and recently it has been shown that this action can be inhibited by the neutralizing anti-IL-6 antibody siltuximab in preclinical and clinical studies [65].

A recent study revealed that activation of macrophages by the infusion of antibodies against CD40 may induce macrophage-mediated tumour regression in 30% of cases in both a mouse model for pancreatic cancer and in patients with pancreatic cancer [66, 67].

Since TGF-β is responsible for skin tumour infiltration by macrophages enabling the tumours to escape immune destruction [68], TGF-β seems to be a major player in the formation of the suppressive tumour microenvironment. Blockade of TGF-β has been shown to enhance tumour vaccine efficacy, but at this moment the exact mechanism has not been unravelled yet [69]. Since CCL2 plays a major role in the recruitment of TAMs, anti-CCL2 would be a logical step in preventing this recruitment. However, it seems that anti-CCL2 does not prevent the influx of TAMs [70]; this could be due to the inability to reach an adequate dosage of anti-CCL2 in the tumour microenvironment to counteract the influx of TAMs.

4.5. Regulatory T Cells. Tregs are a population of CD4+ T cells with a central role in the prevention of autoimmunity and the promotion of tolerance via their suppressive function on a broad repertoire of cellular targets [71]. Characteristic of human Tregs is the expression of CD25 (IL-2 receptor-α chain), forkhead box P3 (Foxp3) transcription factor, glucocorticoid-induced TNF-receptor-related-protein (GITR), lymphocyte activation gene-3 (LAG-3), cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), and a downregulation of CD127 (IL-7R); however, all these markers are not truly Treg specific [72]. Tregs can be divided into natural Tregs and adaptive Tregs. Natural Tregs are important in the suppression of autoreactive T cells that slip through the selection processes, and therefore natural Tregs maintain peripheral tolerance against self-antigens preventing autoimmunity. In humans, these cells represent 2–5% of total circulating CD4+ T cells in peripheral blood [73]. Adaptive Tregs arise from naive T cells and are triggered by suboptimal antigen stimulation and stimulation with TGF-β. Adaptive Tregs can be subdivided into IL-10 secreting Tregs type I (Tr1 cells), TGF-β producing Tregs (Th3 cells), or IL-35 secreting Tregs (iTr35 cells). These cells are characterized by the secretion of immune suppressive cytokines directly inhibiting T cells and converting DCs into suppressive APCs [74]. This contagious spread of suppressive capacity, mainly mediated by IL-35 from Tregs, to other T cells is called infectious tolerance [75].

Tregs infiltrate human cancers, and their prevalence in tumour-infiltrating lymphocytes is much higher than their proportion in peripheral blood, constituting 20% or more of tumour-infiltrating CD4+ lymphocytes [76]. Elevated levels of Tregs have been identified in blood of cancer patients, compared with normal individuals, and their presence predicts for poor survival [77]. In mesothelioma patients, elevated levels of Tregs have also been identified in pleural fluid, with a clear patient-to-patient variability [78].

Natural Tregs are derived in the thymus and migrate into the periphery. It has been proposed that Tregs need to be activated and/or expanded from periphery and bone marrow if needed. Since 25% of CD4+ T cells in the bone marrow function as Tregs, it has been suggested that the bone marrow plays an active role in humoral and cellular immune regulation.

TAA-specific Tregs accumulate in the peripheral lymphoid organs and at the tumour side. However TAA-specific Tregs are also found in the bone marrow, suggesting that after activation Tregs can migrate back to the bone marrow and induce T cell tolerance before these cells enter the circulation [79]. Although exact mechanisms are not fully explored, it has been shown that CCR4+ (receptor for CCL22) Tregs migrate toward tumour microenvironments expressing CCL22 [12]. Also CD62L and CCR7 have been described as important homing markers on Tregs [80]. CD62L is critical for the migration of Tregs to draining lymph nodes. CCR7 is

expressed by a majority of Tregs and is essential in homing to lymphoid organs and microenvironments expressing CCL19 (the ligand for CCR7) [81].

As MDCSs and TAMs, Tregs have several pathways that limit antitumour responses.

(i) Direct cell-cell interaction between Tregs and target cells is important for tolerance induction by Tregs [82]. These target cells include CD4+ and CD8+ effector cells, B cells, NK, T cells, DCs, and monocytes/macrophages. The cell-cell binding leads to apoptosis by activation of programmed cell death ligands (PDLs), the release of perforin [83] and granzyme-A or B [36] and by reducing the proliferation through upregulation of intracellular cyclic AMP [84, 85].

(ii) Tregs produce themselves or induce other cells to secrete immunosuppressive cytokines such as IL-10, IL-35, and TGF-β to blunt immune responses [86], but also other molecules produced by Tregs like carbon monoxide [87] and galectins [88] are reported to play roles in suppression. However, the relative importance of the individual inhibitory factors is dependent on the target disease and experimental model.

(iii) Tregs can inhibit antitumour effector NK and NK T cells via membrane-bound TGF-β [89]. The binding of membrane-bound TGF-β on Tregs to TGF-β-receptor on target cells leads to the activation of intracellular pathways, which eventually leads to the downregulation of the NKG2D-receptor on NK and NKT cells.

(iv) CTLA4+ Tregs induce the expression of indoleamine 2,3-dioxygenase (IDO) in APCs, a potent regulatory molecule mediating the catabolism of the essential amino acid tryptophan into the proapoptotic kynurenine, which is toxic to neighbouring T cells [90].

(v) Tregs are forming aggregates around DCs to prevent contact between DCs and T cells and in this way disturb the induction of the adaptive immune response by preventing proper antigen presentation [91, 92].

(vi) Treg aggregation leads to decreased upregulation of CD80 and CD86 on immature DCs and downregulation of these molecules on mature DCs [93]. These phenomena are antigen specific and dependent on lymphocyte function-associated antigen 1 (LFA-1) and CTL-associated protein 4 (CTLA-4) [22].

(vii) Tregs induce B7-H4 expression by APCs, a member of the B7 family that negatively regulates T-cell responses [94].

(viii) Expression of both ectoenzymes CD39 and CD173 on Tregs can hydrolyse pericellular ATP/AMP into the cAMP or the immunosuppressive nucleoside adenosine [95].

(ix) Binding of lymphocyte activation gene 3 (LAG3) on Tregs to the MHC class II molecules expressed on immature DC suppresses DC maturation [96].

(x) Activated Tregs, which express more high-affinity IL-2R than conventional T cells, may absorb IL-2 from the microenvironment and therefore starve effector T cells that need IL-2 to survive [97].

However, none of these mechanisms can explain all aspects of suppression. It is probable that various combinations of several mechanisms are operating, depending on the milieu and the type of immune responses.

4.6. Targeting Tregs. Owing to the significant role of Tregs in the failure of immune surveillance and immunotherapy, many attempts to deplete Tregs or inhibit their function in cancer patients have been studied. Many of the strategies to reduce Tregs target CD25, which makes up the alpha subunit of the IL-2R, that is present on the surface of Tregs and activated cells. An engineered recombinant fusion protein of IL-2 and diphtheria toxin (denileukin diftitox [Ontak]) and other CD25-directed immunotoxins (daclizumab, LMB-2, RFT5-SMPT-dgA) have been investigated for Treg depletion, which seems to kill selectively lymphocytes expressing the IL-2 receptor. However, early human trials have not proven that this approach results in tumour regression and have shown that these strategies may not adequately deplete Foxp3+ Tregs and may also deplete antitumour effector cells [98–101]. Other possible approaches to reduce immunosuppression of Tregs are via CTLA-4 blockade (e.g., ipilimumab) [102, 103], anti-GITR agonism [104], and vaccination against Foxp3 [105], and some other suggested approaches, such as the inhibition of IDO, TGF-β, ectonucleotidase (expressed by Tregs and generates immunosuppressive adenosine), or the activation of other agents such as OX40 or Toll-like receptor 8 have not yet proven to be beneficial. IL-7 administration was shown to increase T cell numbers and decrease the Treg fraction in humans [106]; on the contrary, other reports have shown that IL-7 leads to the development of Tregs [107, 108]. In conclusion, there are many conflicting results in abrogating the action of Tregs, and thus it is unclear which approach holds promise for cancer treatment.

Low-dose cyclophosphamide (CTX) prevents the development and functionality of the Tregs [109]; the mechanism behind this effect, however, is not completely understood. We investigated the effect of CTX on immune suppression, and the combination of CTX and DC-based immunotherapy was studied in a murine MM model [110]. Our data showed that metronomic administration of low-dose CTX has a strong immune-modulating effect *in vivo*. This is currently tested in a clinical trial in mesothelioma patients (ClinicalTrials.gov Identifier: NCT01241682). Tregs can be significantly reduced in mice with antimurine CCL2/CCL12 monoclonal antibodies, resulting in significant reductions in Treg cells in the spleens and tumours. Using these antibodies, the tumour microenvironment was also drastically altered. This resulted in a significant improvement of immunotherapy [69]. Sorafenib has been

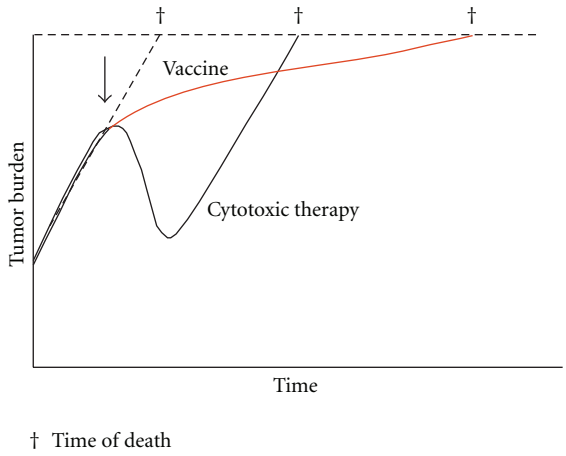

Figure 3: Tumour growth is a dynamic biologic process, that is, the net result of cells dividing and other cells dying. Intrinsic tumour biology, as well as extrinsic factors such as therapies, affects the tumour's growth rate. However, chemotherapy only affects the tumour growth rate while it is being administered, which may result in a dramatic but transient response. Following discontinuation of chemotherapy, the growth rate returns to its pretreatment slope, driven by the underlying biology of the tumour. Immunotherapy (red line), on the other hand, can alter the biology of the host by inducing an active antitumour immune response including a memory response. This may not cause an immediate or dramatic change in tumour burden, but continued cumulative slowing pressure on tumour growth rate, especially if started early in the disease course, which may lead to substantially longer overall survival. The arrow indicates the initiation of treatment; cross indicates time of death from cancer [48]. (Figure used with permission from author.)

proven cytotoxic for Tregs, although the pathway is not fully understood. Sorafenib treatment is associated with a decrease in frequency of Treg cells without influencing the function of peripheral immune effector cells [111]. Recently, p300 was found to be an important target for modulation of host Foxp3+ Treg functions, and an inhibition of p300 using a small molecule inhibitor, C646 (p300i), impaired Foxp3 acetylation and inhibited Treg function [112].

4.7. Immune-Adjuvant Therapies. An alternative approach to immunotherapy is to enhance the intrinsic activity of the immune system. In this field, ipilimumab was proven to be active in metastatic melanoma [113]. Ipilimumab is a monoclonal antibody against cytotoxic T-lymphocyte antigen (CTLA)-4. It is normally expressed at low levels on the surface of naïve effector T cells but is upregulated on the cell surface when there is a long-lasting and strong stimulus via the T cell receptor (TCR). CTLA-4 then competes with CD28 for CD80/CD86 on APCs, effectively shutting off TCR signalling, and thereby serves as a physiologic "brake" on the activated immune system [114]. Ipilimumab prevents this feedback inhibition, resulting in an unabated immune response against the tumour. The side effects of this therapy, however, can be significant due to the downregulation of tolerance to patient's own normal tissue, and colitis is often seen in patients [115]. In mesothelioma, preclinical models have been well described, and a phase II trial is currently ongoing in Italy [26].

Other preclinical approaches are the Toll-like receptor (TLR) ligands to activate DCs [116] or TLR7 agonist to induce systemic, CD8+ T-cell, and type-I IFN antitumour responses [117].

5. Need for Revising Response Evaluation in Immunotherapy

Immunotherapy represents a new class of agents in the treatment of mesothelioma. As seen for Sipuleucel-T in prostate cancer and ipilimumab in melanoma, improvement in the overall survival of patients was seen; however, the agents did not change initial disease progression. Even a transient worsening of disease manifested either by progression of known lesions or the appearance of new lesions can be seen, before disease stabilizes or tumour regresses.

Commonly accepted treatment paradigm, however, suggests that treatments should initially decrease tumour volume, which can be measured using CT scan. Also, progression-free survival is increasingly used as an alternative end-point of studies. This seems to be unfortunate for immunotherapy, which may initiate an immune response that ultimately slows the tumour growth rate, resulting in longer survival, but not a decrease in tumour volume on CT or an increased progression-free survival (Figure 3). Future trials are currently planned to investigate these hypotheses; however, clinicians at this moment may need to reconsider how to measure success of their immunotherapeutic approach [48].

6. Summary

In conclusion, the role of the immune system in mesothelioma is vast. The tumour uses villainous tricks to evade immune surveillance and harnesses itself against the immune system. Immunotherapy tries to modulate this immune system to strengthen the antitumour effect, which is unfortunately hampered by these defence mechanisms from the tumour. At this moment, MSDCs, TAMs, and Tregs seem to be the key players in this process, but undoubtedly extended research will eventually unravel this complex interplay of cells and will reveal more cell types and/or subtypes. Targeting these defence mechanisms could be the key to fully unleash the potential of immunotherapy. Since several cell types are responsible for tumour survival, probably combination therapy targeting multiple cell types will be necessary. It is thrilling that the immunotherapy has been established in several tumour types as a proven therapy in recent years and that many trials are ongoing with promising results. In mesothelioma, the first steps have been made, and, using the accumulating knowledge, immunotherapy will hopefully prove to be an effective treatment.

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Review Article

Macrophages in Tumor Microenvironments and the Progression of Tumors

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Macrophages are widely distributed innate immune cells that play indispensable roles in the innate and adaptive immune response to pathogens and in-tissue homeostasis. Macrophages can be activated by a variety of stimuli and polarized to functionally different phenotypes. Two distinct subsets of macrophages have been proposed, including classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages express a series of proinflammatory cytokines, chemokines, and effector molecules, such as IL-12, IL-23, TNF-α, iNOS and MHCI/II. In contrast, M2 macrophages express a wide array of anti-inflammatory molecules, such as IL-10, TGF-β, and arginase1. In most tumors, the infiltrated macrophages are considered to be of the M2 phenotype, which provides an immunosuppressive microenvironment for tumor growth. Furthermore, tumor-associated macrophages secrete many cytokines, chemokines, and proteases, which promote tumor angiogenesis, growth, metastasis, and immunosuppression. Recently, it was also found that tumor-associated macrophages interact with cancer stem cells. This interaction leads to tumorigenesis, metastasis, and drug resistance. So mediating macrophage to resist tumors is considered to be potential therapy.

1. Introduction

Macrophages were initially described by Elie Metchnikoff, who won the Nobel prize in 1905 because of his identification of phagocytes and his phagocytosis theory [1]. Since then, much progress has been made in revealing the mechanisms underlying macrophage activation and roles that macrophages play in our bodies. Today, it is well established that macrophages are important innate immune cells with essential roles in the primary response to pathogens, normal tissue homeostasis, presentation of foreign and self-antigens following infection or injury, resolution of inflammation, and wound healing.

Macrophages exist in almost all tissues and play important roles in the maintenance of tissue homeostasis. In mature adults, macrophages differentiate from peripheral blood monocytes, which develop from common myeloid progenitor cells. These cells are identified as granulocyte/macrophage colony-forming units (GM-CFUs) in the bone marrow. In response to a macrophage colony-forming factor, GM-CFUs sequentially give rise to macrophage colony-forming units (M-CFUs), monoblasts, and pro-monocytes. Subsequently, they move into the peripheral blood and differentiate into monocytes. Finally, the monocytes migrate into different tissues and replenish the populations of long-lived tissue-specific macrophages, such as alveolar macrophages and kupffer cells [2, 3]. However, not all tissue macrophages are differentiated from monocytes. It has been reported that Langerhans cells in the skin and microglial cells in the brain, which are tissue-resident macrophage populations that are radiation resistant, seem to be maintained through local proliferation, and recent studies indicate that these cells initially develop from M-CFU in the yolk sac of the developing embryo [4].

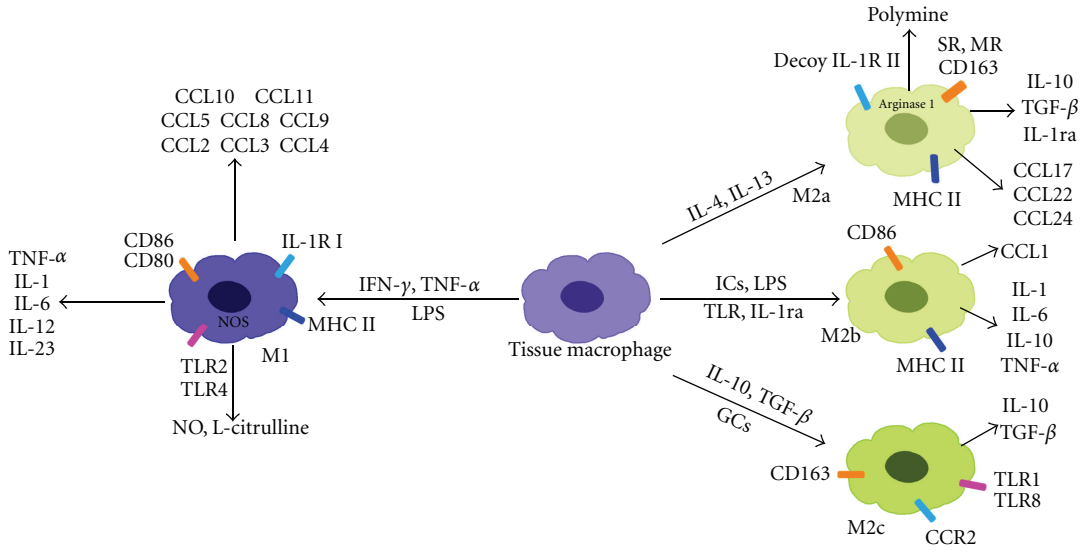

Figure 1: Classically and alternatively activated macrophages. Classically polarized macrophages are activated by LPS, IFN-γ, or TNF-α. Alternatively polarized macrophages can be further divided into M2a, M2b, and M2c macrophages. IL-4 and IL-13 always activate macrophages to be M2a macrophages. The main difference between M1 and M2a macrophages is in their metabolism of L-arginine. In M1 macrophages, L-arginine is metabolized into L-citrulline and NO by NOS2, while in M2a macrophages, it is metabolized into polyamine and urea by arginase 1. M2b macrophages are activated by immune complexes, TLRs, or IL-1ra. Finally, M2c macrophages are polarized by IL-10. All of the phenotypes express a series of different cytokines, chemokines, and receptors.

Macrophages, like other immune effector cells, can have multiple subtypes and take on various phenotypes depending on the microenvironment. By analogy to the Th1/Th2 classification, two distinct states of polarized activation for macrophages have been proposed: the classically activated (M1) macrophage and the alternatively activated (M2) macrophage subsets [5]. M1 macrophages arise following stimulation with the Th1 cytokine interferon-γ (IFN-γ) alone or in concert with bacterial moieties, such as lipopolysaccharide (LPS) or cytokines (e.g., tumor necrosis factor-α (TNF-α)) [6] (Figure 1). In contrast, M2 macrophages are polarized by distinct stimuli and can be further subdivided into M2a, M2b, and M2c macrophages. M2a macrophages are stimulated by the Th2 cytokines IL-4 or IL-13, and M2b macrophages are induced by immune complexes (ICs), LPS, TLRs, or the IL-1 receptor antagonist (IL-1ra). Finally, M2c macrophages are induced by IL-10, transforming growth factor-β (TGF-β), or glucocorticoids (GCs) [7] (Figure 1). M1 macrophages secrete high levels of proinflammatory cytokines (e.g., TNF-α, IL-1, IL-6, IL-12, and IL-23) and increase their concentrations of superoxide anions, oxygen radicals, and nitrogen radicals [8, 9]. Most of these agents can increase their killing activities. Furthermore, M1 macrophages can express high levels of MHC I and class II antigens and secrete complement factors that facilitate complement-mediated phagocytosis [10]. M1 macrophages can also secrete high levels of inducible nitric oxide synthase (iNOS; NOS2) to promote arginine metabolized into nitric oxide and citrulline [11]. Conversely, M2 macrophages always express the scavenger receptor (SR), the mannose receptor (MR), and IL-10, which lead M2 macrophages to mainly participate in parasite clearance, tissue remodeling, immune modulation, and tumor progression [9]. In this paper, we will discuss the characteristics of differentially polarized macrophages and explore the role of tumor-associated macrophages (TAMs) in tumor progression.

2. Properties of Polarized M1 and M2 Macrophages

Macrophages can exert different properties when polarized with distinct inducers. Differential cytokine production is a key feature of polarized macrophages. When stimulated with IFN-γ, M1 macrophages secrete high levels of IL-12 and IL-23 but low levels of IL-10 [3, 12–14]. In contrast, M2 macrophages express high levels of IL-10 but low levels of IL-12 and IL-23 [14, 15]. Because of their different cytokine profiles, these polarized macrophages have distinct functions. For example, the IL-12 produced by M1 macrophages can promote the differentiation of Th1 cells, which can improve antigen phagocytosis [12, 16]. IL-23, which is also secreted by M1 macrophages, is associated with the development and expansion of Th17 cells, which can secrete high levels of IL-17 and contribute to inflammatory autoimmune pathologies [17, 18]. In addition, the IL-10 expressed by M2 macrophages can promote the production of IL-4 and IL-13 by Th2 cells [19]. IL-4 is a major promotor of wound healing because it can activate arginase, which contributes to the production of the extracellular matrix. The differential metabolism of L-arginine provides a means of distinguishing the two macrophage activation states. M1 macrophages upregulate iNOS to catabolize L-arginine to nitric oxide (NO) and

citrulline, but M2 macrophages induce arginase 1, which metabolizes arginine to ornithine and polyamines, which are precursors necessary for collagen synthesis and cellular proliferation [20].

Differentially polarized macrophages can also express different chemokines. For instance, LPS and IFN-γ induce macrophages to express chemokine (C–X–C motif) ligand 9 (CXCL9), CXCL10, and CXCL5 through the activation of the transcription factor IFN regulatory factor-3 (IRF-3), which results in IFN-β expression and subsequent STAT1 (signal transducer and activator of transcription 1) activation. These proinflammatory chemokines can promote the recruitment of Th1, Tc1, and NK cells, which can improve their capacity for intracellular pathogen killing [10]. In contrast, M2 macrophages inhibit CXCL9, CXCL10, and CXCL5 by downregulating NF-κB and STAT1 [21, 22]. M2a macrophages induced by IL-4 and IL-13 promote the expression of Chemokine (C–C motif) ligand 24 (CCL24), CCL17, and CCL22. These chemokines can specifically combine with Chemokine (C–C motif) receptor 3 (CCR3) and CCR4, which accelerate the recruitment of eosinophils, basophils, and Th2 cells, to lead to a type II response. M2b cells always secrete CCL1, which combines with CCR1 to promote the infiltration of eosinophils, Th2, and regulatory T cells. These cells exert immune regulation and drive the Th2 response. Finally, in M2c macrophages, IL-10 induces CXCL13, CCL16, and CCL18, which can combine with CXCR5, CCR1, and CCR8 to promote the accumulation of eosinophils and naïve T cells, which play a prominent role in suppressing immune responses and promoting tissue remodeling [10]. In considering the above information, we found that the role of chemokines expressed by different macrophages is in accordance with the cytokines they express. The M1-derived chemokines are important for killing intercellular pathogens, whereas the M2-derived chemokines promote the recruitment of the leukocytes involved in tissue repair and remodeling.

Heterogeneity and plasticity are important features of macrophages. Under different stimuli, macrophages can polarize into different phenotypes. However, these phenotypes are not stable. Several in vivo studies have demonstrated that the phenotype of an activated macrophage population can change over time. For example, during tumor progression, the macrophage phenotype changes from classically activated to alternatively activated [23]. In contrast, the macrophage phenotype changes from M2 to M1 in obesity [24]. However, clarification on whether this phenotypic alteration is the result of a dedifferentiation of the original macrophages back to the resting state or the migration of a new population of macrophages into the tissue site that replace the original cells is still needed. In vitro investigations have clearly shown that polarized macrophages (M1 or M2) change their expression profile according to changes in stimuli [2]. Therefore, macrophages could repolarize in response to changes in the local microenvironment, allowing them to shape the local inflammatory milieu to adapt to outside stimuli. High plasticity is an important characteristic of macrophages and contributes to the development of certain disorders.

As we mentioned above, macrophages in different microenvironments play different roles. Next, we will discuss the role of TAMs in tumor progression.

3. The Role of TAMs in Tumor Progression

A tumor, as defined by Wills, is "an abnormal mass of tissue, the growth of which is uncoordinated with that of the normal tissues and persists after the cessation of the stimuli which evoked the change." Tumors are composed of proliferating tumor cells and stromal cells, including endothelial cells, inflammatory cells, and fibroblasts [25]. In the 1970s, it was found that TAMs, as the predominant leukocyte, play a key role in tumor growth [26].

The role of TAMs in tumors is still controversial. It has been reported that in colorectal tumors TAMs are proinflammatory, and play an antitumor role, which leads to a good prognosis [27, 28]. One possible reason is that the M1 TAMs promote colon tumor cell expressing galcetin-3 which further induce more TAMs infiltration and lead to an amplification immune response to destruct tumor cells [28]. On the other hand, TAMs express a series of proinflammatory cytokines such as IFN-γ, IL-1, and IL-6, which activate type-1 T-cell associated with antitumor immune responses [27]. However, in most tumors such as breast, prostate, ovarian, cervical, lung carcinoma, and cutaneous melanoma, TAMs are considered to be antiinflammatory and correlated with a poor prognosis. Epidemiological studies have suggested that a macrophage-rich microenvironment will promote an aggressive tumor with a high metastatic potential [29]. Therefore, many scholars have further studied the function of TAMs in tumorigenesis. In the present study we will focus on how the anti-inflammatory TAMs influence the progression of tumors.

TAMs exhibit an M2-like phenotype because they express a series of markers, such as CD163, the Fc fragment of IgG, C-type lectin domains, and heat shock proteins [30–32]. On the other hand, the tumor microenvironment includes a number of chemoattractants, such as IL-4, IL-13, TGF-β, and IL-10, all of which lead to the adoption of an M2 phenotype [33]. TAMs orchestrate various aspects of cancer, such as tumor progression, angiogenesis, tumor growth, actual metastasis, immunosuppression, matrix deposition, and remodeling (Figure 2).

3.1. Monocyte Recruitment. TAMs are differentiated from monocytes by a number of chemoattractants that are produced by tumor cells and stromal cells. For instance, tumor-derived chemokine CCL2, formerly known as monocyte chemotactic protein (MCP), is critical for the recruitment of macrophages [34, 35]. CCL2 is produced by tumor cells, fibroblasts, and macrophages, and high CCL2 levels are correlated with increased numbers of TAMs and a poor prognosis [36]. Other chemokines, such as CCL3, CCL4, CCL5, CCL7, CCL8, CXCL12, and cytokines, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and IL-10, are also reported to promote macrophage recruitment [14, 37–39]. In addition,

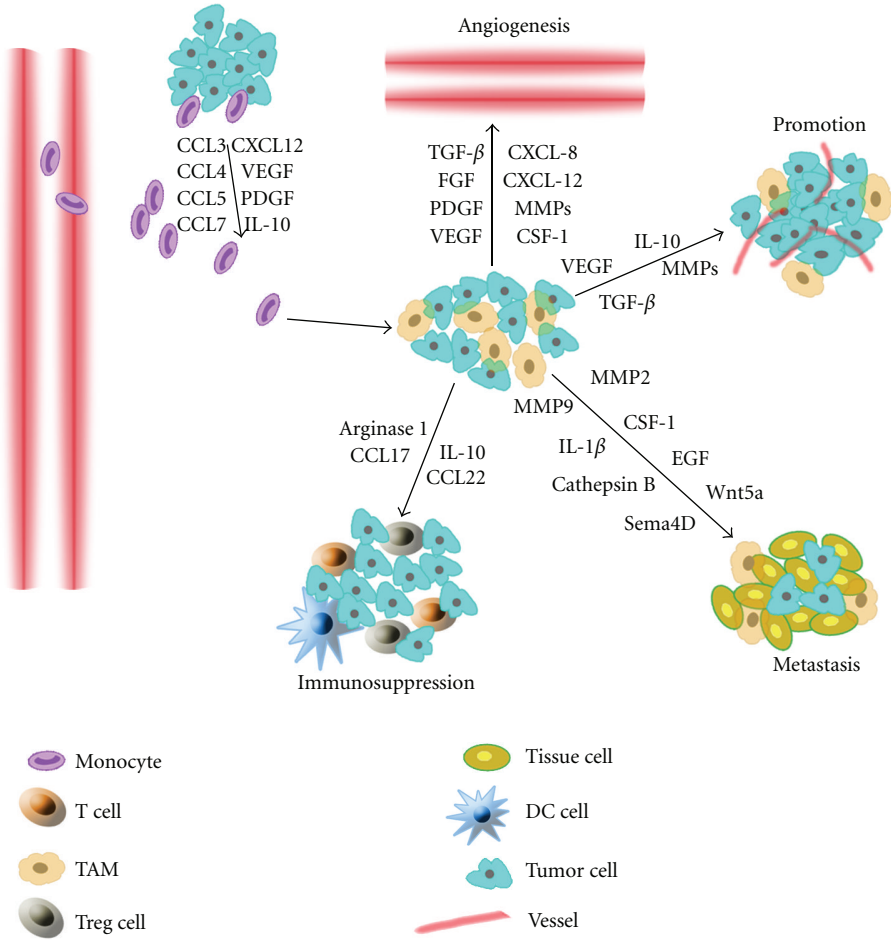

Figure 2: TAM functions in tumor progression. Tumor cells and stromal cells, which produce a series of chemokines and growth factors, induce monocytes to differentiate into macrophages. In the tumor, most macrophages are M2-like, and they express some cytokines, chemokines, and proteases, which promote tumor angiogenesis, metastasis, and immunosuppression.

another group of monocyte chemoattractants, the alarmins, have been reported to promote the recruitment of monocytes and other myeloid cells [40]. For example, the high mobility group box protein 1 (HMGB1), which is one of the molecules released by dying tumor cells, is found in the necrotic areas where TAMs preferentially reside. Other alarmins, such as S100A8, S100A9, serum amyloid A3 (SAA3), and fibronectin, have also been reported to attract $CD11b^+$ myeloid cells [41].

3.2. TAMs and Angiogenesis. Tumors do not grow beyond 2-3 mm^3 and cannot metastasize unless they are vascularized [42]. It is well known that the growth and spread of malignant tumors requires angiogenesis, the process by which new blood vessels sprout from the existing vasculature. Accumulating evidence indicates that TAMs play an important role in regulating angiogenesis. Bingle and his colleagues demonstrated that TAMs present within a solid tumor significantly contribute to the initiation of angiogenesis. In the absence of TAMs, the tumor cells produce the necessary stimuli to initiate tumor angiogenesis, but the initiation is delayed [43]. More recently, Zeisberger et al. found that depleting TAMs with clodronate encapsulated in liposomes (clodrolip) could reduce blood vessel density in the tumor tissue [44]. These results validate the idea that TAMs present in the tumor microenvironment promote angiogenesis in tumors.

How do the TAMs regulate angiogenesis? It has been reported that TAMs are able to modulate and induce neovascularization and support functions. When TAMs are activated, they can express a broad repertoire of substances (including growth factors, cytokines, proteases, and chemokines) to promote angiogenesis. For instance, TAMs release growth factors such as VEGF, PDGF, transforming growth factor β (TGF-β), and a member of the FGF family, which can promote angiogenesis in many tumors, such as gliomas, squamous cell carcinomas of the esophagus, and breast, bladder, and prostate carcinomas [14, 36, 45]. In addition, Aharinejad et al. found that the overexpression of colony-stimulating factor 1 (CSF-1) can enhance the recruitment of TAMs, which accelerates tumor development and malignant progression in the mammary epithelium of MMTV-PyMT mice [46]. Lin and colleagues found that, when inhibiting the expression of CSF-1 or its receptor with short-interfering

RNA (siRNA) in mice model, macrophage infiltration and vascularity are decreased compared to their CSF-1 counterparts [47, 48]. Moreover, TAM-derived proteases, such as matrix metalloproteases (MMP-1, MMP-2, MMP-3, MMP-9, and MMP-12), plasmin, and urokinase plasminogen are also beneficial to angiogenesis. MMP-9 is one of the most important proteases that degrade the extracellular matrix (ECM) and further release other growth factors to stimulate angiogenesis [49, 50]. MMP-2 expression is also increased in several tumors, which is correlated with the nodal status and tumor stages [51].

TAMs have been found to accumulate in hypoxic regions of tumors, which are characterized by low-oxygen tension. As TAMs adapt to the hypoxic microenvironment, they can express more proangiogenic genes, such as VEGF, pFGF, CXCL8, and glycolytic enzymes, whose transcription is controlled by the transcription factors HIF-1 and HIF-2 [42]. In addition, it has been reported that the HIF-1-dependent chemokine CXCL-12 acts as a potent chemoattractant that promotes endothelial cell infiltration when specifically combined with its sole receptor, CXCR4 [52].

3.3. TAMs and Lymphangiogenesis. Lymphangiogenesis is the initial step in the generalized spread of tumor cells, which predicts a poor clinical prognosis. TAMs promote the lymphangiogenesis mediated by VEGF-C and VEGF-D via VEGFR3 [53]. It has been reported that VEGF-C and VEGF-D are produced not only by tumor cells but also by TAMs. In human cervical cancer, the VEGF-C released by TAMs plays a novel role in peritumoral lymphangiogenesis and the subsequent formation of lymphatic metastases [54]. However, in bladder cancer, VEGF-C expression was positively associated with both lymphangiogenesis and angiogenesis, while VEGF-D was associated only with lymphangiogenesis [55]. In addition, TAMs can express lymphatic endothelial growth factors to promote lymphangiogenesis [54, 56].

Recently, Maruyama and colleagues found that $CD11b^+$ macrophages physically contribute to lymphangiogenesis under pathological conditions and that bone marrow-derived $CD11b^+$ macrophages express lymphatic endothelial markers, such as LYVE-1 and Prox-1, under inflamed conditions in the corneal stroma of mice [57]. These findings suggest that macrophages induce lymphangiogenesis in two different ways, either by transdifferentiating and directly incorporating into the endothelial layer or by stimulating the division of preexisting local lymphatic endothelial cells [58].

3.4. TAMs and Tumor Growth. In addition to promoting angiogenesis and lymphangiogenesis, TAMs also play a pivotal role in tumor growth. It has been demonstrated that TAM infiltration is positively correlated with the proliferation of tumor cells in several tumors, such as breast cancer, endometrial cancer, and renal cell cancer [59, 60]. Macrophages cocultured with tumor cells could secrete a series of substances which facilitate tumor cell proliferation [42]. Additionally, macrophage depletion studies have proven that TAMs are essential for tumor growth [61].

MMP9, which was mentioned earlier as a primary factor promoting angiogenesis, also plays an important role in tumor growth. The cytokine IL-23 is considered to promote tumor incidence and growth by upregulating MMP9, thereby stimulating inflammatory responses [62, 63]. Moreover, TAMs limit the cytotoxicity of the microenvironment, which helps tumor growth. Because TAMs are M2-like, they can secrete large amounts of IL-10, which can suppress cytotoxic T-cell activity by inhibiting Th1 cells while simultaneously inhibiting NK and lymphokine-activated killer cell cytotoxicity [42].

Recently, a notable paper reported that in melanomas, TAM-derived adrenomedullin is involved in angiogenesis and tumor growth. It was found that the adrenomedullin derived from TAMs interacts with its receptors on endothelial cells to promote tumor growth via a paracrine loop through the activation of the eNOS signaling pathway similar to the angiogenesis cytokine VEGF [64]. On the other hand, TAM-derived adrenomedullin can influence macrophages themselves in an autocrine manner. The inhibition of adrenomedullin receptors on TAMs impairs angiogenesis and tumor growth [65, 66].

3.5. TAMs and Tumor Metastasis. Tumor metastasis is an important marker in determining the severity of cancer. Tumor cells metastasize through the blood and lymphatic vessels, which leads to the formation of ectopic tumors. These tumors present a great therapeutic challenge and result in a poor prognosis [67]. More than 20 years ago, with mouse models, Gorelik et al. found that TAMs promote tumor metastasis. After intravenous injection of murine tumor cells, the macrophage population increased during the formation of lung tumor nodules [68]. Subsequently, Wyckoff and colleagues demonstrated a synergistic relationship between breast cancer cells and TAMs in cell migration [67].

Coffelt et al. found that both TAM and tumor cells migrated frequently when they were in close proximity by multiphoton microscopy. Further study revealed that the epidermal growth factor (EGF) released by TAMs interacted with the CSF-1 released by tumor cells to promote the migration of the tumor cells [41]. Accumulating studies have verified that the malignant cells always move next to the TAMs, which appear to help malignant cells during intravasation [69]. Pawelek and Chakraborty even proposed that when cancer cells fuse with migratory bone marrow-derived cells, they provide the driving force behind the dissemination process [70].

On the other hand, TAMs influence the microenvironment, which can also promote tumor cell invasion. Hagemann and colleagues demonstrated that coculturing TAMs with tumor cells can promote the expression of MMPs, especially MMP2 and MMP9, in TNF-α-dependent manner [71]. Both MMP2 and MMP9 help degrade the proteins in the extracellular matrix to promote metastasis [42]. In addition, Seth et al. showed that MMP7 could also promote tumor metastasis through converting the receptor activator of nuclear factor κB ligand (RANKL) [72, 73]. Other macrophage-derived molecules, such as IL-1β, cathepsin B, Wnt5a,

and semaphorin 4D (Sema4D), have also been reported to promote tumor metastasis [41].

Recently, it has been reported that macrophage-derived microRNA (miRNA) also regulated tumor invasion. Yang et al. found that oxesomes containing miR-233 shuttle between macrophages and breast tumor cells. miR-233-regulating tumor invasion is considered to work through the mef2c-β-catinen pathway [74]. This research indicated that cell-to-cell interaction is not only restricted in protein but also provides us a new research direction in future.

3.6. TAMs and Immunosuppression. Tumor immunosuppression is a well-established mechanism for the regulation of tumor growth. Several studies have reported that TAM-derived cytokines and proteases, such as TGF-β, IL-10, and arginase 1, make a significant contribution to immunosuppression [75–77]. For instance, TGF-β has a crucial immunosuppressive role in both the innate and the adaptive arms of the immune response. In the innate immune response, TGF-β promotes tumor-associated macrophage polarization to an M2-versus-M1 phenotype, which further promotes TGF-β production and deepens immunosuppression [78]. TGF-β also inhibits the cytolytic activity of natural killer (NK) cells expressing the activating receptor NKG2D, further resulting in a poor antitumor response [79, 80]. In addition, TGF-β decreases dendritic cells (DCs) migration and increases apoptosis, which decreases antigen presentation and dampens the adaptive immune response [81, 82]. In the adaptive immune response, TGF-β promotes $CD4^+$ T cells differentiation into Th2 cells rather than Th1 cells, which promotes a less efficient antitumor immune response [83]. TGF-β also inhibits the $CD8^+$ T cells antitumor activity by suppressing the expression of several cytolytic genes, including the genes encoding granzyme A, granzyme B, IFN-γ, and FAS ligand [79, 84]. Furthermore, TGF-β promotes tumor growth by the maintenance of Treg cell differentiation, which inhibits the antitumor response [79].

IL-10, an important cytokine in the tumor microenvironment, is expressed by TAMs, $CD8^+$ T-cells, and tumor cells. IL-10 is commonly regarded as an anti-inflammatory, immunosuppressive cytokine that favors tumor escape from immune surveillance. TAM-derived IL-10 acting in an autocrine circuit suppresses the expression of IL-12, a potential antitumor cytokine [85]. Several studies have reported that TAM-derived IL-10 prevents the maturation of DCs in situ but increases the differentiation of macrophages, which decreases antigen presentation [76, 86]. IL-10 can also inhibit the release of the cytotoxic cytokine IFN-γ, which is the main factor that stimulates naïve T-cell differentiation, to promote immune evasion [87]. Furthermore, it has been reported that IL-10 decreases the ability of epidermal APCs to present tumor-associated antigens for the induction of antitumor immune responses in a spindle cell tumor system [88]. However, not all agree with that IL-10 leads to immunosuppression. Some articles reported that IL-10 possesses some immunostimulating properties, which play important roles in antitumor response [89–91]. For example, in NSCLS stage I, it is found that the more infiltrating $CD8^+$/IL-10^+ cells there are, the longer the overall survival will be [89]. So the role of IL-10 in tumor microenvironment is still controversial. To make it clear may take a huge forward for tumor therapy.

Arginase 1, the molecular marker for M2 macrophages, is highly expressed in tumors. In recent years, it has been demonstrated that arginase 1, which primarily metabolizes L-arginine into polyamine and proline, causes dysregulation of the T cell receptor (TCR) signal and subsequently induces $CD8^+$ T cell unresponsiveness [77, 92]. In addition, several studies have reported that arginase 1 activation is associated with H_2O_2 production by myeloid-derived suppressor cells (MDSCs), which present class I-restricted epitopes directly to $CD8^+$ T cells and inhibit their release of IFN-γ through the contact-dependent production of H_2O_2 [93]. However, the concrete mechanism underlying the H_2O_2 generation following arginase 1 activation is not clear and may be linked to the synchronous activation of a different NOS isoform [92].

Finally, several studies have found that chemokines also play an important role in immunosuppression. Chemokines, such as CCL17 and CCL22, can prevent the infiltration of cytotoxic T-cells but promote that of Treg and Th2 cells [38, 94]. TAM-derived CCL18 has the ability to recruit naïve T-cells, which induces T-cell anergy [14]. CCL-2 and CCL-5, which were mentioned previously as chemoattractants of monocytes to tumors, induce suppression of T-cell responses [76]. Further studies have shown that TAM-induced immunosuppression is correlated with the activation of transcription factors, such as STAT3, STAT6, and NF-κB, but the specific mechanism still needs to be explored [41].

3.7. Interaction between TAM and Cancer Stem Cells (CSCs). Over the past 5 to 10 years, it has been found that a specific subpopulation of tumor cells has distinct stem cell properties in tumors. These cells are defined as cancer initiating cells or cancer stem cells (CSCs). A CSC has the ability to initiate tumorigenesis by undergoing self-renewal and differentiation [95, 96]. However, stromal cells, such as fibroblasts and immune cells, are also known to play important roles in tumor progression [97]. Therefore, research on the relationship between CSCs and stromal cells has become an exciting area of focus.

TAMs, as the dominant immune cell components, are considered to be closely related to CSCs in position. Several studies have reported that TAMs are always found distributed around CSCs, and the number of infiltrating TAMs has been positively correlated with the histological grade of the malignancy and the number of CSCs found [98, 99]. Furthermore, Yi, et al. found that the production of CSC-derived chemoattractants, including CCL2, CCL5, VEGF-A, and NTS, in glioma tissue was much higher than in adhesive glioma cells (AGCs), which promotes the infiltration of macrophages. However, when a specific antibody to the chemoattractants was used, the migration of the macrophages decreased. These results indicate that CSCs play a more dominant role in recruiting macrophages than AGCs [100]. At approximately the same time as the publication

of these results, another paper reported that CSCs in glioma tissue induce macrophage infiltration and polarize the macrophages into an M2 phenotype because the macrophages secreted a large number of cytokines, such as TGF-β1, IL-10, and IL-23. In addition, M2 macrophages could induce T-cell anergy and therefore immunosuppression in agreement with what we mentioned previously [101]. Both of these articles indicated that CSCs play a leading role in macrophage infiltration and polarization.

Recently, other articles have demonstrated that macrophages can also influence the characteristics of CSCs, which promote tumorigenesis and metastasis. For example, Jinushi and colleagues found that CSCs could specifically stimulate TAMs to express the downstream factor milk-fat globule epidermal growth factor VIII (MFG-E8), which has been identified as a growth factor involved in phagocytosis, angiogenesis, and immune tolerance. MFG-E8 induces CSCs to form tumors and develop antitumor drug resistance through the STAT3 and hedgehog signaling pathways [102]. Okuda et al. found another novel phenomenon: highly metastatic breast CSCs upregulate the expression of hyaluronan synthase HAS2, which correlates with tumorigenicity and tumor progression in several cancers. The interaction between CSCs and TAMs through hyaluronan stimulates the secretion of PDGF-BB, which in turn activates stromal cells to secrete the FGF7 and FGF9 that stimulate CSC proliferation, self-renewal and metastasis in the bone [103].

All of these studies indicate that macrophages promote CSC proliferation and metastasis. However, the investigation into the interaction between macrophages and CSCs is still at an early stage. More in-depth research requires our joint efforts.

4. Potential Therapies Targeting TAMs

Accumulating studies have demonstrated that the density of TAMs is associated with a poor prognosis, suggesting macrophages as a target for clinical therapy [104–107]. As early as in 1970s, Dolph Adams has raised the point that macrophage mediated tumor cytotoxicity (MTC). It is considered that macrophages can be activated through two steps: The basic step is through the cytokines and other small molecules and the secondary signal is supplied by either antibody or LPS/endotoxin/TLR stimulants. Both of the signals can activate MTC and resist tumor activity [108]. Today it is considered through the following steps such as antimacrophage infiltration, antiangiogenesis, and converting M2 to M1 to resist macrophage-mediated tumor activity.

Several studies reporting the use of "antimacrophage" approaches have primarily focused on counteracting monocyte chemokines and receptors as anticancer targets [10, 14, 36, 42]. In the murine model for breast cancer, macrophages were recruited by the tumor cell-derived chemokine CCL5. After treatment with the receptor antagonist met-CCL5, both the number of infiltrating macrophages and the size of the tumor were significantly reduced [109]. In addition, some studies have shown that Trabectedin, a natural product derived from the marine organism *Ecteinascidia turbinata*, has a specific cytotoxic effect on human macrophages and TAMs in vitro [110]. Recently, it was reported that pharmacological drugs, such as zoledronic acid combined with sorafenib, enhance antitumor effects by depleting the macrophage population [111]. Other pharmacological drugs, including thalidomide, linomide, pentoxifyline, and genistein, have also been shown to inhibit macrophage infiltration and reduce tumor size [112, 113].

As we mentioned earlier, tumors do not grow beyond 2-3 mm^3 unless they are vascularized, so inhibiting angiogenesis is also a good therapeutic approach. Several studies revealed that anti-VEGF-A with Avastin/bevacizumab or other neutralizing antibodies can both inhibit the infiltration of macrophages and enhance the activity of antiangiogenic therapies by preventing TAMs from secreting additional pro-angiogenic factors [114, 115].

As M1 macrophages induce proinflammatory response which protects body from injury, converting the M2 macrophages into M1 is also considered to be a better potential therapy. Several articles have reported that activation of TLRs stimulates M1-polarized macrophage response, which induce the activation of proinflammatory program [116]. In a mouse model, Guiducci et al. found that CpG plus anti-interleukin-10 receptor antibody promptly switched infiltrating macrophages infiltrate from M2 to M1 and triggered innate response debulking large tumors [117]. SHIP1 is a crucial phosphatase in the conversion from macrophage M1 to M2 functions. Therefore, pharmacological modulators of this phosphatase that can promote the infiltration of M1 macrophages and inhibit M2 macrophages, thereby enhancing the antitumor effects of M1 cytotoxicity, are under investigation [14, 118].

In addition, accumulating studies report using macrophages as natural vectors to deliver therapeutic molecules to the neoplastic site [14, 41, 119]. For instance, intratumoral injection of macrophages transfected with an IL-12-expressing recombinant adenoviral vector can enhance the number of $CD4^+$ and $CD8^+$ cells and reduce tumor growth and metastasis [120]. Moreover, Siveen and Kuttan found that paclitaxel, a plant-derived diterpenoid, can stimulate macrophages to express high levels of NO, TNF-α, and IL-1β. Through the increased levels of these substances, paclitaxel can enhance tumor cell cytotoxicity and restore IL-12 production by macrophages in tumor-bearing mice [36]. Recently, it was reported that an anti-PD-L1 antibody, which blocks the PD-1/PD-L1 pathway, can improve macrophage-mediated T-cell activation in HCC in vivo and has progressed to a phase I clinical study [121, 122]. Perhaps this antibody will be an effective drug in the future.

5. Conclusion

Heterogeneity is one of the most important characteristics of macrophages. In different diseases, macrophages can be polarized into different phenotypes. In most tumors, macrophages are considered to be polarized into the M2 phenotype. TAMs express a series of cytokines, chemokines, and proteases to promote angiogenesis, lymphangiogenesis,

tumor growth, metastasis, and immunosuppression. Recently, it has also been reported that TAMs interact with CSCs, which facilitate tumorigenicity, metastasis, and drug resistance. Taken together, these findings indicate that targeting macrophages in the tumor microenvironment may provide more efficacious novel therapies for future tumor management.

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Review Article

Novel Anti-Melanoma Immunotherapies: Disarming Tumor Escape Mechanisms

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The immune system fights cancer and sometimes temporarily eliminates it or reaches an equilibrium stage of tumor growth. However, continuous immunological pressure also selects poorly immunogenic tumor variants that eventually escape the immune control system. Here, we focus on metastatic melanoma, a highly immunogenic tumor, and on anti-melanoma immunotherapies, which recently, especially following the FDA approval of Ipilimumab, gained interest from drug development companies. We describe new immunomodulatory approaches currently in the development pipeline, focus on the novel CEACAM1 immune checkpoint, and compare its potential to the extensively described targets, CTLA4 and PD1. This paper combines multidisciplinary approaches and describes anti-melanoma immunotherapies from molecular, medical, and business angles.

1. Introduction

The interplay between cancer cells and the host immune system displays an intriguing, dynamic battle for life. The current dogma on tumor progression under immune pressure is of the three "E"s: elimination, equilibrium, and escape [1]. In the first phase, the innate and adaptive immune system tracks and eliminates nascent tumor cells (immune surveillance). If not all cancer cells are eliminated, the second phase is equilibrium between cancer and the immune system, in which for a while, sometimes lasting years, the tumor remains dormant. This equilibrium, however, is temporary as genetic instability of cancerous cells together with continuous pressure of immune cells gradually shapes the immunogenicity of the tumor, transforming it into poorly immunogenic. This process, called immune editing, leads eventually to tumor escape and thereby progression into clinically evident disease. The immune system thus suppresses tumors on the one hand while promoting it on the other hand, by selecting and encouraging poorly-immunogenic variants (reviewed in [1–3]). The mechanisms of tumor escape are numerous. They include alteration of the features of the tumor cells themselves (up-regulation of anti-apoptotic molecules and of cytotoxic determinants and downregulation of antigen presentation MHC molecules), secretion of cytokines that inhibit effective immune response (e.g., VEGF, IL-10, and TGFβ), and the induction of an immuno suppressive environment by indoleamine 2,3 dioxygenase (IDO) or via recruitment of inhibitory immune cells (Treg, MDSC, NKT, iDC, and macrophages) [4–6].

We will focus here on metastatic melanoma, which is an excellent example for the above mentioned model, as it is highly immunogenic and responds to immunotherapy [7]. Malignant melanoma is a main cancer-related cause of death in people below thirty. It is the most rapidly increasing malignancy in Western population in terms of incidence and is currently the sixth most common cancer in the USA, displaying high mortality rate, surpassed only by lung cancer [8, 9]. As surgery is beneficial only for localized (primary) melanoma, continuous efforts are made to find effective

immunotherapies for metastatic melanoma (MM). Systemic treatments include the administration of nonspecific immune-stimulating cytokines [7], immunization with cancer cells or molecules [10], adoptive T cell transfer [11], the recently developed small inhibitors of melanoma oncogenes [12], and blocking antibodies against inhibitory immune molecules [13]. Accumulating data proved that melanoma induces both innate and adaptive immune responses and that immune cells home to and infiltrate melanoma masses. However, the avidity of these cells is probably low, due to low cell number, low cytotoxic potential, or inhibitory microenvironment [14–17]. We will here describe promising immune treatments that aim to enhance the naturally occurring anti-melanoma immune response.

2. Anti-Melanoma Immunotherapies

2.1. Anti-CTLA4 (Ipilimumab and Tremelimumab). CTLA-4 is an inhibitory molecule expressed on T cells undergoing activation, which functions to prevent prolonged activation signals. T cells are activated by two sequential signals: antigen recognition (TCR binding to antigen/MHC on APCs) and costimulation (e.g., CD28 interaction with B7.1 or B7.2 on APCs). CTLA-4 competes with CD28 on the binding of B7 and, when upregulated, inhibits CD28-dependent proliferation and activation and instead leads to cell cycle arrest, decreased cytokine production, and IDO secretion from APCs [18, 19]. Noteworthy, it was reported that CTLA-4 is also expressed by various tumor cells [20] and in a Wnt-dependent manner in melanoma [21]. Stimulation of tumor-expressed CTLA-4 with soluble ligands or agonistic mAb leads to induction of apoptosis [20, 21] as well as inhibition of proliferation and secretion of angiogenic cytokines [22]. These observations point out that CTLA-4 exerts nonimmune-related functions when expressed by nonlymphoid cells. It could also reflect a yet undefined mechanism by which tumors achieve an "immune escape" phenotype and actively suppress, evade, and avoid T cell immunity [23].

Complete knockout of CTLA-4 is lethal, and mice suffer from massive lymphoproliferation and organ destruction [24, 25]. However, preclinical studies showed that blocking of CTLA-4 results in anti-tumor activity and tumor regression in many mice tumor models (prostate, breast, lymphoma, melanoma) [26–29], which paved the way for clinical studies. Two anti-CTLA-4 monoclonal antibodies, generated by different companies, were tested in clinical trials in MM patients: Tremelimumab (Pfizer) and Ipilimumab/Yervoy (Bristol Myers Squibb), but only the latter was successful in phase III studies. Based on its ability to prolong survival of previously treated as well as untreated MM patients [30, 31], Ipilimumab gained European Union (2010) [32] and FDA (March 2011) approval.

Two exciting phase III studies tested the clinical effects of Ipilimumab in advanced MM patients. In the first, 676 participants from 125 different medical centers that were already treated with standard treatments received either Ipilimumab, gp100 vaccine, or the combination of both, in a randomized, double-blind manner. Treatment with Ipilimumab improved median overall survival rates (10.0 and 10.1 months in the Ipilimumab-treated groups as compared with 6.4 months in the gp100-only treated group). The percentages of the patients who responded to Ipilimumab in the two groups were very limited (complete response in ~1% and partial response in 5–10%), but the effects of response were long-lasting in the majority of the responders [30]. In the second trial [31], 502 patients that were not previously treated received either dacarbazine (DTIC, standard care chemotherapy) or Ipilimumab in combination with dacarbazine in a double-blind, placebo-controlled manner. In this experiment, Ipilimumab increased overall survival rates from 9.1 to 11.2 months and 3-year survival from 12.2% to 20.8%. Adverse effects, mainly immune related in the skin and gastro-intestinal track, accompanied nearly all patients in the two trials, with about half of the patients suffering from severe adverse effects in the second trial and several severe immune effects-related deaths in the first trial. These exciting results thus also exhibit the complicity of specifically manipulating immune responses.

2.2. Anti-PD1 (MDX-1106 and CT-1101). PD-1, as CTLA-4, is an inhibitory receptor belonging to the CD28 superfamily of immune-regulatory receptors. However, while CTLA-4 expression is limited to T cells, PD-1 has a broader expression profile and is expressed on activated T, B and several myeloid cells. PD-1 (programmed death 1) downregulates T cell function (proliferation, cytokine secretion, and cytolysis of target cells) by delivering negative signals upon binding to its ligands, PD-L1 and PD-L2 (reviewed in [33]). PD-L2 expression is restricted to APCs (dendritic cells and monocytes) [34–36], and it is involved in tolerance of T cells to environmental (e.g., orally administrated) antigens [37]. PD-L1, on the contrary, is expressed by multiple normal and cancerous tissues and confers peripheral tolerance from "self" antigens [38, 39]. Upon normal levels of antigen exposure, PD-1 functions as a "gate keeper" to attenuate immune responses (reviewed in [40]). The importance of PD-1 is manifested in PD-1-deficient mice, which suffer from auto-immunities [41, 42]. Upon abnormal antigen exposure levels (chronic viral infection, caner) however, this immune tolerance becomes a stumbling block, as PD-1 delivers "veto" signals for CTLs, a response which renders tumor cells protected from cytotoxic immune cells and hampers anti-tumor immune interventions, such as vaccinations and ACT [40]. PD-L1 is upregulated in cancerous cells *in vitro* by immune cytokines, which are critical for T cell functioning, such as IFNγ [43], which may even positively feedback to enhance immune tolerance *in vivo*. Indeed, PD1-deficient mice exhibit enhanced anti-tumor T cell responses towards solid and hematopoietic tumor, including melanoma, these mice survive longer and the tumors are regressed [39, 44, 45] and tumor transduced to overexpress PD-L1 grew more aggressively *in vivo* [46]. Blocking the PD1/PD-L1 pathway delays tumor progression [39, 44, 47–49] and adoptive transfer of tumor-specific PD-1-deficient T cell receptor transgenic T cells can reject tumors [43]. In melanoma patients, PD-L1 is expressed on melanoma cells and the levels of PD-L1 expression positively correlate with overall

survival [50]. PD-1 is upregulated in CD-8$^+$ T cells from melanoma patients during the metastatic (III, IV) stages of disease [50] and this upregulation may be associated with T cell dysfunction [51].

In order to block the inhibitory PD-1/PD-L1 pathway, two different anti-PD-1 monoclonal inhibitory antibodies were generated, MDX-1106 (BMS-936558) [52] and CT-011 [53]. Phase I clinical studies with each of the antibodies proved their safety, well-tolerated administration, and limited toxicity (though in both of them the maximum tolerated dose was not reached) and provided pharmacokinetic data [52, 53]. In these clinical experiments, MDX-1106 (fully human antibody) was assayed in 39 patients with advanced melanoma, colorectal cancer, prostate cancer, non-small-cell lung cancer and renal cell carcinoma [52]. In the CT-011 study (humanized antibody), 17 patients were included, with leukemia, lymphoma, or multiple myeloma [53]. Clinical benefit was observed in both experiments [52, 53] and clinical responses correlated with the extent of PD-L1 expression on tumors [52]. Phase II clinical studies with MDX-1106 are ongoing with biweekly administration in metastatic non-small-cell lung cancer, renal cell carcinoma, prostate cancer and metastatic melanoma. They show limited toxicity, good tolerance (maximum tolerated dose (MTD) was not reached) and anti-tumor activity with 37.5% objective response in the total patients cohort (including 3 melanoma patients). One of the most impressive results was that all responses were highly durable and were still ongoing when publishing these preliminary results [54]. Phase II clinical trials with CT-011 are also ongoing (http://www.clinicaltrials.gov/). Two other antibodies of the PD-1 pathway are under clinical development (currently recruiting participants for phase I studies): MK-3475 (anti-PD-1) and MDX-1105-01 (anti PD-L1) (http://www.clinicaltrials.gov/). The combination of anti-PD-1 and anti-CTLA-4 was tested in murine B16 melanoma model and found to be more effective in tumor regression as compared to each of the blocking antibodies alone [55]. A phase I clinical trial involving the two antibodies is ongoing, as well as a trial that combines MD-1106 with melanoma vaccines (http://www.clinicaltrials.gov/).

2.3. Comparison between Anti-CTLA-4 and Anti-PD-1. The different features of CTLA-4- as compared with PD-1-deficient mice [25, 42] and the synergism of anti-CTLA-4 and anti-PD-1 treatment in animal models [55] suggest that they act in distinct, non-redundant pathways. Though not enough experimental data using anti-PD-1 has been collected, the MTD of anti-PD-1 was not yet reached, and the drugs were not compared in a randomized manner, anti-PD-1 seems to evoke less severe and less frequent adverse effects as compared with anti-CTLA-4 [52]. These differences may be attributed to the different cellular targets of the drugs. Anti-CTA-4 targets a peripheral interaction, between T cells and APCs. Thus, it is expected to cause general stimulation accompanied by adverse effects. The exact mechanism of action of MDX-1106 is not known. However, as it blocks the interactions of PD-1 with both PD-L1 and PD-L2 [52], it may act not only in the periphery but also within the tumor sites, interfering with T cell/tumor cell interactions and evoking specific, localized stimulation. In searching for localized immune modulators, which act within the tumor milieu and whose manipulation will not lead to severe autoimmunity, we have studied the roles of CEACAM1 in melanoma (Figure 1).

2.4. CEACAM1 as a Novel Immunotherapeutic Target. Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1, CD66a), a member of the Ig superfamily, is a broadly expressed, multifunctional, cell-cell adhesion molecule [56, 57]. While not expressed in normal melanocytes [58], it is neoexpressed by the vast majority of melanoma specimens (unpublished observation) and is elevated during the histopathological progression of metastatic melanoma [59]. CEACAM1 is considered as an independent, highly significant marker for the development of melanoma metastases and poor survival [60]. Accumulating *in vitro* evidence suggests that it is not merely a marker but also confers cancerous characteristics to melanoma cells and thus may actively participate in the etiology of melanoma [58, 61]. In the immune system, CEACAM1 acts as an inhibitory molecule that blocks proliferation and cytotoxic activity of T cells [62–64] and NK cells [62, 65–70] via ITIM sequences and the recruitment of SHP-1 and SHP-2 phosphatases [69, 71, 72]. Supporting this immune-inhibitory role, the expression of CEACAM1 on target cells, including melanoma, protects them from being eliminated *in vitro* by NK and T cells [62, 64, 69]. We have recently reported that melanoma cells that have survived an *in-vitro* T cell attack actively increase CEACAM1 expression in an IFNγ-dependent manner [64] and that this elevation enhances the protective effect against subsequent immune attacks [63]. Moreover, we could identify CEACAM1-positive NK cells in lymph nodes infiltrated with CEACAM1-positive melanoma cells, but not with CEACAM1-negative melanoma cells [69]. These data suggest a potentially novel tumor escape mechanism that could be used by CEACAM1-positive melanoma cells to evade elimination by transferring CEACAM1 to the attacking immune cells. Indeed, transfer of CEACAM1 was observed *in-vitro*, although it was considerably less efficient than transfer of CEACAM5 [73]. Importantly, patient-derived melanoma infiltrating lymphocytes [64] and circulating T and NK cells from melanoma patients [68] synthesize and express functional CEACAM1 [64, 68, 69, 74], which renders them susceptible to CEACAM1-mediated inhibition and may thus contribute to cancer progression. We have observed over-expression of CEACAM1 by circulating cytotoxic lymphocytes in other diseases, including ankylosing spondilitis and bare lymphocyte syndrome type I [65, 67, 68], as well as on decidual lymphocytes obtained from CMV-infected pregnancies [62], all occurring due to yet to be defined mechanisms. These may be related to aberrant immune stimulation or to abnormal development of immune cells [68].

Based on these findings, we have developed a high-affinity murine monoclonal antibody against human CEACAM1 [75]. Anti-CEACAM1 does not act on CEACACM1-positive cells in *cis* (i.e., does not interfere with general cellular processes such as proliferation and apoptosis).

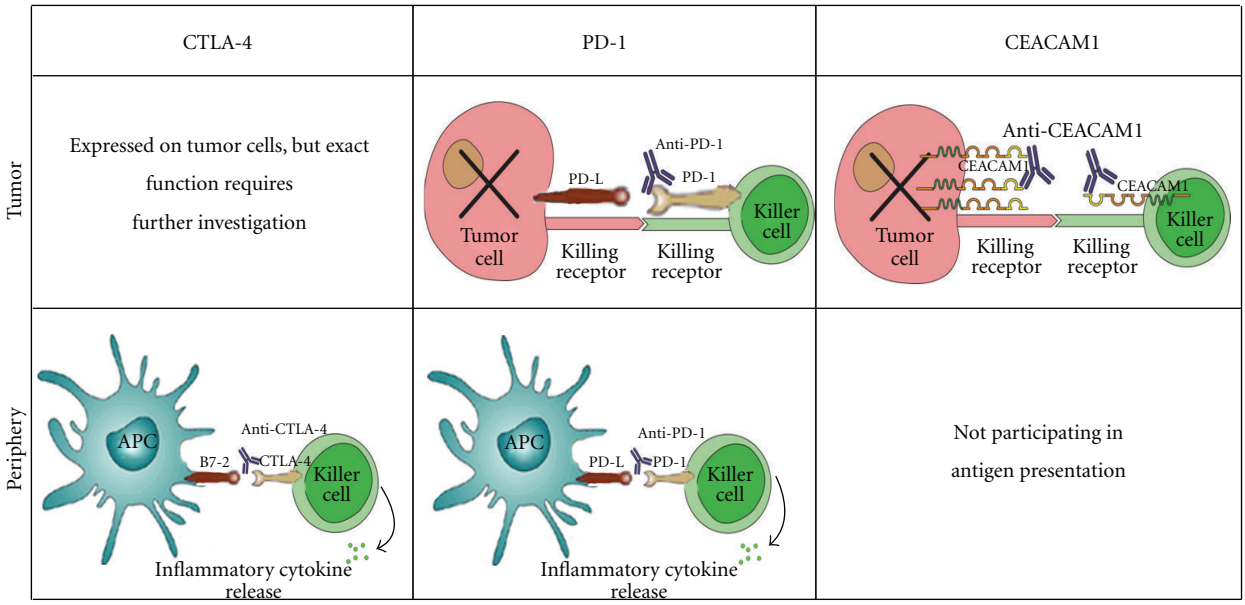

Figure 1: Sites of action for selected immune modulators. Anti-CTLA-4 targets the interactions between T cells and APCs in the periphery and thus is prone to evoke general stimulation accompanied by frequent adverse effects. Anti-PD-1 may block peripheral interactions, as anti-CTLA-4, but also the interactions between tumors and infiltrating immune killer cells, and thus may evoke both general and localized stimulation. CEACAM1 is expressed both on immune killer cells and on the tumor target, and anti-CEACAM1 is expected to act specifically at the interface between these two and to evoke low autoimmunity.

Rather, it acts in *trans*, binding both T cells and melanoma, to efficiently relieve the CEACAM1-dependent inhibition of T cell cytotoxicity. Therefore, the mechanism of action *in vivo* of anti-CEACAM1 strongly depends on the endogenous immune system and its ability to recognize the target cells in an antigen-restricted manner, thereby reducing the risk of adverse effects stemming from generalized non-specific immune stimulation. We showed that anti-CEACAM1 renders melanoma cells susceptible to elimination by T cells, both *in-vitro* and in a human-melanoma xenograft murine model, which maintains antigen-restricted recognition [75]. Indeed, we have previously shown that abolishment of CEACAM1 with polyclonal anti-CEACAM antibodies does not induce a nonspecific T cell function [64].

Several lines of evidence pointed to the potential high specificity of anti-CEACAM1 to the cancerous state and to its potentially low risk of evoking adverse effects: (a) staining of normal tissue micro-array with anti-CEACAM1 proved only limited staining of luminal cells of some secretory ducts. These patterns are substantially more restricted than staining patterns of other FDA-approved therapeutic antibodies, such as Erbitux; (b) the anti-CEACAM1 mAb does not elicit complement-dependent cytotoxic effect nor non-specific T cell activation; (c) the anti-CEACAM1 mAb is not an agonistic antibody and is therefore probably incapable of exerting direct functional effects on CEACAM1-positive cells. Rather, it is an antagonistic antibody, whose effects depend on antigenic recognition between T cells and their targets; (d) the immune-inhibitory homophilic CEACAM1 interactions are expected to take place only in the tumor, and not during earlier stages of the elicited immune response, such as antigen presentation. CEACAM1 homophilic interactions occur between CEACAM1-positive cancer cells and CEACAM1-positive tumor infiltrating lymphocytes, which are late-effector lymphocytes. Thus, blocking of CEACAM1 is expected to enhance the immune response only within tumor sites and only in the context of antigen-restricted recognition. These exciting results mark anti-CEACAM1 as a potential specific and safe (compartmentalized to the tumor vicinity) novel immunotherapeutic modality (Figure 1). Another important advantage of CEACAM1-directed therapy is that patient selection would be based on the presence of CEACAM1 on tumor tissue. It should be noted that CEACAM1 is expressed in 60–80% of metastatic melanoma cases, which suggests that the majority of metastatic melanoma patients would benefit from anti-CEACAM1 antibodies. The anti-CEACAM1 approach is developed by cCAM BioTherapeutics, and first-in-man clinical trials are anticipated in the near future.

2.5. Adoptive T Cell Transfer (ACT). Adoptive cell therapy with *ex vivo* cultured T cells, developed by Rosenberg and his colleagues in the National Cancer Institute, is currently the most promising immunotherapy for MM patients, yielding 50–70% objective response rates [76, 77]. It is based on the isolation of bulk T cell masses from resected melanoma, their *ex-vivo* expansion by about 1000-fold (reaching about 50×10^9 cells), and their reinfusion to the patient following lymphodepleting nonmyeloablative chemotherapy, which eliminates endogenous competitor immune cells [76, 77].

Table 1: Current clinical trials in melanoma using monoclonal antibodies.

Company	Antibody	Target	Function	Status
	Antagonistic Abs.			
Bristol-Myers Squibb	Ipilimumab (Yervoy)	CTLA-4	Relieve immune block	Approved
Bristol-Myers Squibb	MDX-1106[1]	PD-1	Relieve immune block	Phase II (completed)
Curetech Ltd. (Israel)	CT-011	PD-1	Relieve immune block	Phase II (recruiting)
Merck	MK-3475	PD-1	Relieve immune block	Phase I (recruiting)
Bristol-Myers Squibb	MDX-1105-01	PD-L1	Relieve immune block	Phase I (recruiting)
	Agonistic Abs.			
Bristol-Myers Squibb	BMS-663513	4-1BB	Stimulate T cells	Phase II (completed)
Pfizer	CP870,893[2]	CD40	Stimulate T cells	Phase I (recruiting)
Tolerx	TRX518	GITR	Inhibit T regs	Phase I (on hold)
Portland Providence Medical Center	Anti-OX40	OX40	Stimulate T cells	Phase II (not open yet)

[1] Additional phase I studies are ongoing, in combination with Ipilimumab or with melanoma vaccines.
[2] Together with melanoma vaccine and an immune stimulant called Oncovir poly IC : LC (one phase I study) or with Tremelimumab (another phase I study).

Recently, we have shown that T cells derived from enzymatic digestion of resected tumors (rather from multiple small fragments) yield high numbers in culture, which enable to shorten their *ex-vivo* culturing period [78]. We and others have shown that responding patients were treated with TIL that spent less time in culture [78, 79]. Indeed, Young TIL cultures were successfully established for nearly 90% of MM patients, and overall response rates reached 50% [78]. The main disadvantages of ACT are that the generation of TIL cultures presents a technical challenge and is labor, cost and time consuming [76, 77]. Attempts to overcome several of these limitations by the usage of genetically-engineered rather than endogenous T cells were presented in two clinical trials. These trials, in which T cells were modified to overexpress TCR directed against melanoma antigen (MART-1 or gp100), yielded modest response rates (12–30%) but proved the feasibility of the method [80, 81]. Engineering T cells with chimeric antigen receptors (CARs), which recognize tumor cells in a MHC-independent manner and endow increased T cell activity [82], have been tested in preclinical studies in melanoma [83]. Recently, Peng and his colleagues have shown that over-expression of the murine chemokine receptor CXCR2 on T cells improves their homing to melanoma and tumor regression in mice model [84], suggesting that endowing T cells with improved chemotaxis capabilities to tumor sites may also enhance ACT.

2.6. Additional Antibodies. Additional immune-modulatory molecules that have gained scientific attention and are now under clinical development are OX40 (CD134), CD40, GITR, and 4-1BB (CD137) (http://www.clinicaltrials.gov/) (Table 1, also reviewed in [85]).

3. Cancer Immunotherapy: Business Angle

For decades, cancer immunotherapy has been neglected by drug development companies, which were deterred by the field unfavorable track record. Moreover, in order to achieve clinical proof of concept, immunotherapy requires a unique development strategy that involves long-term follow-up and randomized controlled studies. This stems in part from the fact that tumor shrinkage is less common with immunotherapy drugs coupled with the long period required for mounting a systemic immune response. [86]. Lastly, combining immune-modulating drugs with chemotherapy regimens was perceived as counterproductive.

This sentiment has gradually been changing following clinical validation with cancer vaccines and immunomodulatory antibodies. Of particular importance were FDA approvals for Sipuleucel-T and Ipilimumab, based on survival benefit in prostate cancer and melanoma, respectively, [31, 87]. These agents demonstrated unequivocally the value of immunotherapy for cancer in broad unselected populations.

The renewed interest in cancer immunotherapy is best exemplified by recent deals involving clinical and preclinical programs. The growing number of transactions coupled with their lucrative financial terms serves as a testament to the excitement within the industry regarding harnessing the immune system to fight cancer. Although melanoma remains a common indication for immunotherapies, recent data clearly suggests that potential utility for this approach spans well beyond this indication.

4. Deals

In August 2010, Amplimmune licensed MP-224, an Fc-fused PD-L2, to GlaxoSmithKline (GSK). Amplimmune received an upfront payment of $23 M and is eligible to receive $485 M in milestone payments. MP-224 binds and inhibits the immunosuppressive activity of PD-1, a coinhibitory checkpoint on T cells [88]. The fusion protein is expected to be the fourth PD-1 neutralizing agent in clinical testing behind BMS' BMS-936558, Curetech's CT-011, and Merck's MK-3475.

In January 2011, Amgen acquired BioVex, which was developing OncoVex GM-CSF, a genetically modified herpes simplex virus 1 (HSV-1). The deal included $425 M upfront

and $575 M of milestone payments. OncoVex GM-CSF is an oncolytic virus currently in phase III for the treatment of metastatic melanoma. The virus anti-cancer effect involves direct killing of tumor cells followed by immune activation that results from the virus immunogenicity and secretion of GM-CSF to the tumor microenvironment. In its phase II trial, OncoVex GM-CSF exhibited a unique clinical activity profile. Intratumor injection of the virus resulted in tumor shrinkage of injected as well as noninjected lesions. Responses were durable in a substantial portion of patients and overall survival was encouraging. An ongoing phase III trial is expected to generate results in 2012, using a primary endpoint of objective response lasting 6 months or more. Another phase III trial in head and neck cancer has been terminated in 2011.

In July 2011, BMS licensed IPH2102, an antibody targeting KIR receptors, from Innate Pharma. By binding the inhibitory KIR receptors on NK cells, the antibody, currently in phase I, is expected to promote an innate immune response against cancer cells. The deal included an upfront payment of $35 M as well as $430 M in development and commercialization milestones.

In September 2011, Bristol-Myers Squibb acquired ex-US commercialization rights (except in Japan, Korea, and Taiwan) for BMS-936558 from Japan-based Ono Pharmaceuticals. BMS-936558 is a fully human antibody targeting PD-1, for which BMS had originally held US rights. In return, Ono received certain commercialization rights for abatacept (Fc-fused CTLA4) in Japan. BMS-936558 is the most advanced PD-1 inhibitor in clinical testing, currently studied in melanoma, lung, and renal cancer. Initial results with this antibody as a single agent are encouraging [89].

In October 2011, MedImmune (the biologics arm of AstraZeneca) in-licensed two programs in the field of cancer immunotherapy. One deal involved licensing tremelimumab, an anti-CTLA4 antibody from Pfizer. MeDimmune assumed global development rights for Tremelimumab, which failed a phase III trial in melanoma in 2008. Future development will likely be based on pharmacodynamic biomarkers identified retrospectively in the failed phase III study. Pfizer retained the rights to use drug with specified types of combination therapies. Terms of the agreement were not disclosed. A second deal was signed with Portland-based AgonOx, which is developing OX40 agonists for the treatment of cancer. AgonOx is developing Fc-fused OX40 ligand as well as agonist antibodies. A murine antibody against OX40 led to immune activation and tumor shrinkage in a phase I trial (company's web site).

In October 2011, Genesis Biopharma announced a deal with the NIH for patents covering TIL therapy. The deal included an upfront payment of $1.2 M as well as undisclosed milestone payments and royalties. Following the deal, Genesis intends to turn the autologous cell-based treatment, which until now has been given as a service in medical centers, into a commercially available product. The company will offer the treatment, rebranded as Contigo, via several medical centers in the US and plans to manufacture it at a central production facility. The anticipated cost per patient is $120 thousand, similar to that of ipilimumab.

Disclosure

G. Markel is the scientific founder of cCAM Biotherapeutics Ltd.

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Research Article

Immune Responses to RHAMM in Patients with Acute Myeloid Leukemia after Chemotherapy and Allogeneic Stem Cell Transplantation

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Leukemic blasts overexpress immunogenic antigens, so-called leukemia-associated antigens like the receptor for hyaluronan acid-mediated motility (RHAMM). Persistent RHAMM expression and decreasing $CD8^+$ T-cell responses to RHAMM in the framework of allogeneic stem cell transplantation or chemotherapy alone might indicate the immune escape of leukemia cells. In the present study, we analyzed the expression of RHAMM in 48 patients suffering from acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). Furthermore, we correlated transcripts with the clinical course of the disease before and after treatment. Real-time quantitative reverse transcriptase polymerase chain reaction was performed from RNA of peripheral blood mononuclear cells. T cell responses against RHAMM were assessed by tetramer staining (flow cytometry) and enzyme-linked immunospot (ELISPOT) assays. Results were correlated with the clinical outcome of patients. The results of the present study showed that almost 60% of the patients were RHAMM positive; specific T-cells recognizing RHAMM could be detected, but they were nonfunctional in terms of interferon gamma or granzyme B release as demonstrated by ELISPOT assays. Immunotherapies like peptide vaccination or adoptive transfer of RHAMM-specific T cells might improve the immune response and the outcome of AML/MDS patients.

1. Introduction

Approximately 80% of patients with acute myeloid leukemia (AML) reach a complete remission (CR) after chemotherapy. However, half of the patients in CR relapse and only 25% of all AML patients survive more than five years. Therefore, there is a fervent need for novel therapies to treat leukemia including immunotherapeutic approaches. Leukemic blasts overexpress proteins that play an important role in survival and proliferation of the cells. These proteins have been designated leukemia-associated antigens (LAAs). LAAs comprise a broad group of proteins including the receptor for hyaluronic-acid-mediated motility (RHAMM) [1]. $CD8^+$ T-cell responses against RHAMM have been identified in AML patients [2]. Nevertheless, it remains to be elucidated why immunogenic LAAs are expressed but not sufficiently recognized, and why LAA^+ malignant cells are not subjected to lysis through specific $CD8^+$ T cells. Immune escape might become effective through downregulation of LAAs or hampering of the proper function of T cells [3]. Only limited information is available on the expression of LAAs before and after chemotherapy and/or allogeneic stem cell transplantation (allo-SCT). Therefore we investigated here the expression of RHAMM as well as the frequency of RHAMM-specific $CD8^+$ T lymphocytes before and after chemotherapy/allo-SCT. As a future perspective, rather poor

immune responses to RHAMM might be enhanced through immunotherapeutic approaches such as peptide vaccination and adoptive transfer of specific T-cell responses in the context of chemotherapy and allo-SCT [1].

2. Material and Methods

2.1. Blood Samples from Patients and Healthy Donors. We collected 173 peripheral blood samples from 48 patients suffering from AML/MDS after obtaining the patients' informed consent. This study was approved by the local ethical committee. Serial peripheral blood samples were collected at diagnosis, after chemotherapy and/or after allo-SCT with immunosuppression, in CR or during maintenance therapy at sequential time intervals during follow-up and at relapse. Both peripheral blood mononuclear cell (PBMC) and bone marrow mononuclear cell (BMMC) samples were prepared using Ficoll Biocoll separating solution (Biochrom, Berlin, Germany) and cryopreserved according to standard protocols. PBMC samples were obtained from 10 healthy donors and used as negative controls.

2.2. Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RQ-RT-PCR). RNA was isolated from a minimum of 2×10^6 cells using RNeasy plus minikit (QIAGEN, Düsseldorf, Germany). Five hundred nanograms of RNA were reversely transcribed into cDNA using the iScript cDNA synthesis kit (BioRad, Munich, Germany). The reverse transcription (RT) products were diluted with 70 μL of molecular-biology water (Sigma-Aldrich). Nine micro liters was used per well. Primers/probes (TaqMan Gene Expression Assays, Invitrogen) were diluted in TaqMan 2xPCR Master Mix according to manufacturer's instructions. Standard curves for RHAMM and ABL were established for each run of RT-PCR using four dilution steps per gene. Copy numbers were calculated by http://www.endmemo.com/. Reactions were tested in duplicate using the ABI PRISM 7900 sequence detection system (Applied Biosystems) and standard conditions with 40 cycles of amplification in 20 μL of volume.

2.3. Mixed Lymphocyte Peptide Culture (MLPC). MLPC was performed as described elsewhere [4]. Briefly, specific $CD8^+$ T cells were selected from PBMCs and BMMCs by magnetic-activated cell sorting (MACS) columns (Miltenyi). $CD8^-$ fraction was irradiated with 30 Gy and loaded with test or control peptides (20 μg/mL) or cultured with medium alone (no peptide). Peptide sequences of RHAMM and control peptides derived from phosphoprotein-65 of the cytomegalovirus (CMVpp65) and influenza matrix protein (IMP) were ILSLELMKL, NLVPMVATV, and GILGFVFTL, respectively. $CD8^+$ and $CD8^-$ cells were cocultured in a ratio of 1 : 4. MLPC was supplemented with 10 U/mL IL-2 (Sigma Aldrich) and 20 ng/mL IL-7 (Miltenyi) on day 1. Cytotoxic T lymphocytes (CTLs) were harvested on day seven for enzyme-linked immunospot (ELISPOT) assay and/or flow cytometry analysis when sufficient numbers of $CD8^+$ cells were collected.

2.4. Mini-MLPC. The MLPC approach was modified into a mini-MLPC in case that insufficient numbers of $CD8^+$ cells were obtained from MACS separation. Mini-MLPCs were performed in round-bottom 96-well microtiter plates in RPMI-1640 culture medium supplemented with 10% heat-inactivated human AB serum, 10 U/mL IL-2, and 20 ng/mL IL-7. The ratio was maintained as in the MLPC (1×10^4 $CD8^+$ and 4×10^4 $CD8^-$ cells, 1 : 4). Number of cells per well was based on the work by Distler et al. [5]. Proliferation observed in mini-MLPCs was comparable to proliferation in conventional MLPCs.

2.5. ELISPOT for Interferon Gamma (IFN-γ) and Granzyme B. IFN-γ and granzyme B ELISPOT assays were performed as described elsewhere [4] to determine specific recognition of RHAMM peptide-positive target cells according to manufacturer's instructions (BD, San Diego, USA).

2.6. Flow Cytometry Analysis. The frequency of RHAMM-specific T cells was determined by flow cytometry. Tetramer staining was performed as described previously [2]. Briefly, lymphocytes were stained with RHAMM-specific tetramers and subsequently with conjugated antibodies to CD3 and CD8 (BD Biosciences). Fluorescein isothiocyanate (FITC), peridinin-chlorophyll protein (PerCP), and phycoerythrin (PE) were used as fluorochromes. A minimum of 2×10^4 cells were acquired. Flow cytometry was performed on a Calibur cytometer (BD Biosciences). Appropriated isotype controls were included in each experiment. Data were analyzed using the flow cytometry analysis software FlowJo (Tree Star, Inc, USA). The frequency of tetramer $CD8^+$ T cells was considered positive if it was 2-fold or higher than the frequency of $CD8^+$ cells counterstained with a tetramer recognizing an irrelevant peptide.

2.7. Clinical Status of the Patients. The clinical status of patients was obtained from the clinical data base of the Department of Internal Medicine III, University of Rostock. Clinical features of the patients such as chimerism analysis, cytogenetics, HLA, CMV status, and therapy were evaluated by the Department of Internal Medicine III, University of Rostock. The FLT3 status was assessed at the Department of Hematology/Oncology at the University of Greifswald, Greifswald, Germany. AML cases were classified according to the FAB criteria and characterized at the cytogenetic level. CR was defined according to standard criteria.

2.8. Statistical Analysis. Statistical analyses were performed using Stat Graphics Plus 5. The standard Wilcoxon signed-rank test was used for nonparametric comparisons of median expression of RHAMM before and after treatment, as the data were paired and not normally distributed. Mann-Whitney *U*-test was used for nonparametric comparisons of median expression of RHAMM in healthy donors and patients. Statistical significance was considered if the *P* value was <0.05.

3. Results

3.1. Patients' Characteristics. We screened 48 AML/MDS patients in a prospective study. Twenty-one patients received allo-SCT, whereas 27 received only chemotherapy under conventional protocols. Our cohort of patients maintained a ratio of almost 1 : 1 between male ($n = 23$) and female patients ($n = 25$). A normal karyotype was found in 19 patients, and aberrant karyotype in 17 patients, and a complex karyotype in seven patients. The karyotype of five patients was not accessible. There was no significant difference between the age of men and women ($P = 0.5$) at the time of diagnosis. The mean follow-up time of the patients was 272 days (median: 225 days). Nine patients died from leukemia, and three patients from diseases not related to leukemia, that is, encephalitis, pneumonia, and graft versus host disease.

3.2. Expression of RHAMM Transcripts before and after Treatment. Expression of *RHAMM* in the peripheral blood of healthy donors ($n = 10$) was very low (median: 318; range 97–730 *RHAMM* copies/10^4*ABL* copies). In contrast *RHAMM* transcripts were significantly higher in patients before treatment (median: 768; range: 184–36, 160; $P = 0.001$). In order to compare different treatments, patients at diagnosis were split in two groups: patients after chemotherapy alone and patients after allo-SCT (Figure 1). Peripheral blood was collected from 22 AML patients at the time of diagnosis. Thirteen patients were *RHAMM* positive (59%), whereas nine were negative (41%). The expression of *RHAMM* was considered positive when it was higher than the highest value measured in the peripheral blood of healthy donors. There was no significant difference in expression of *RHAMM* in the peripheral blood of patients before treatment according to *FLT3-ITD* status (positive versus negative, $P = 0.89$), gender ($P = 0.66$), or karyotype (normal versus aberrant, $P = 0.29$; normal versus complex, $P = 0.75$; aberrant versus complex, $P = 0.40$).

Furthermore, we aimed to determine the expression of *RHAMM* before and after chemotherapy alone or allo-SCT. Therefore we measured absolute copy numbers of this gene in AML/MDS-diagnosed patients. There was no significant difference before and after treatment, neither by chemotherapy ($P = 0.83$) nor by allo-SCT ($P = 0.28$). However, we observed higher transcript numbers during CR of patients that received allo-SCT when compared to those who received chemotherapy ($P = 0.009$). Furthermore, *RHAMM* was also equally expressed before and after allo-SCT in a cohort of patients in CR. This group also showed higher copy numbers of *RHAMM* after transplantation when compared to the group that was treated with chemotherapy ($P = 0.007$, Figure 1).

3.3. RHAMM-Specific CTLs in Healthy Donors. RHAMM-specific T cells were observed in three of ten healthy donors, that is, in HD 155, HD 663, and HD 005, at low frequencies (0.11%, 0.33%, and 0.12%, resp.) with cells in the $CD3^+CD8^+$gate set 100% (Figure 2(g) with controls

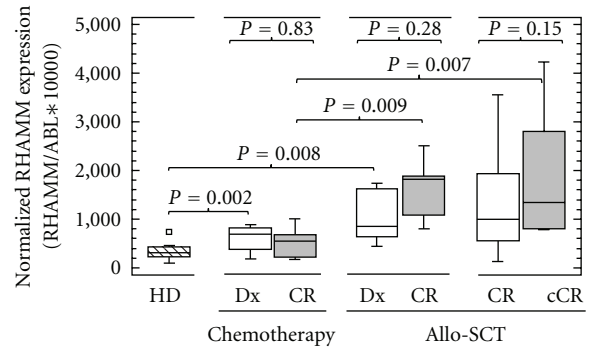

Figure 1: RHAMM expression before and after chemotherapy alone or allo-SCT. Patients at diagnosis had higher copy numbers when compared to healthy donors (chemotherapy group, $P = 0.002$; allo-SCT group, $P = 0.008$). There was no significant difference in expression of *RHAMM* before (white boxes) and after (grey boxes) treatment, neither for chemotherapy alone ($P = 0.83$) nor for allo-SCT ($P = 0.28$), in patients at diagnosis that reached a CR after treatment. Also patients in CR that received allo-SCT showed no difference in *RHAMM* transcripts before (white boxes) and after (grey boxes) transplant ($P = 0.15$). However, patients that received allo-SCT had higher copy numbers when compared to those who received chemotherapy during the CR ($P = 0.009$) or continuous CR (cCR, $P = 0.007$). Medians are shown in the box plots. RHAMM: receptor for hyaluronic acid mediated motility, allo-SCT: allogeneic stem cell transplantation, CR: complete remission, P-value: probability value.

Figures 2(c)–2(f)). Moreover, an activity of these RHAMM-specific CTLs was detected in two healthy donors (HD 155 and 669) as for IFN-γ secretion evaluated by ELISPOT assays (Figure 2(a)). CTLs from healthy donor 669 were not sufficient in number to perform flow cytometry analysis.

3.4. RHAMM-Specific CTLs in Patients. Longitudinal studies of RHAMM-specific $CD8^+$ T cells were only possible with samples of ten patients who overexpressed RHAMM (29/48 patients) and were HLA-A2 positive, as all peptides used in this study were HLA-A2 restricted. The frequency of RHAMM-specific $CD8^+$ T cells in the peripheral blood was measured by flow cytometry during the course of the disease. Furthermore their activation status and potential to kill $RHAMM^+$ malignant cells was assessed by secretion of IFN-γ and granzyme B, respectively. As displayed in Figure 3(c), RHAMM-specific CTLs were detected at a frequency of 1.24% up to 5.62% of all $CD8^+$ T cells, and 0.03% up to 1.14% of all cells in the lymphocyte gate. In ELISPOT assays a general activity of $CD8^+$ T cells (Figures 3(a) and 3(b)) was detected at the time of diagnosis (Dx) and in CR. Thirty days prior to allo-SCT a stronger release of granzyme B by RHAMM-R3-stimulated $CD8^+$ T cells than by unstimulated T cells was detected. After allo-SCT a general silencing of T cells as for release of both IFN-γ and granzyme B was detectable (Figures 3(a) and 3(b)). In accordance with these findings flow cytometry analysis revealed that the number of RHAMM-$tetramer^+$ $CD8^+$ T cells vanished over the time.

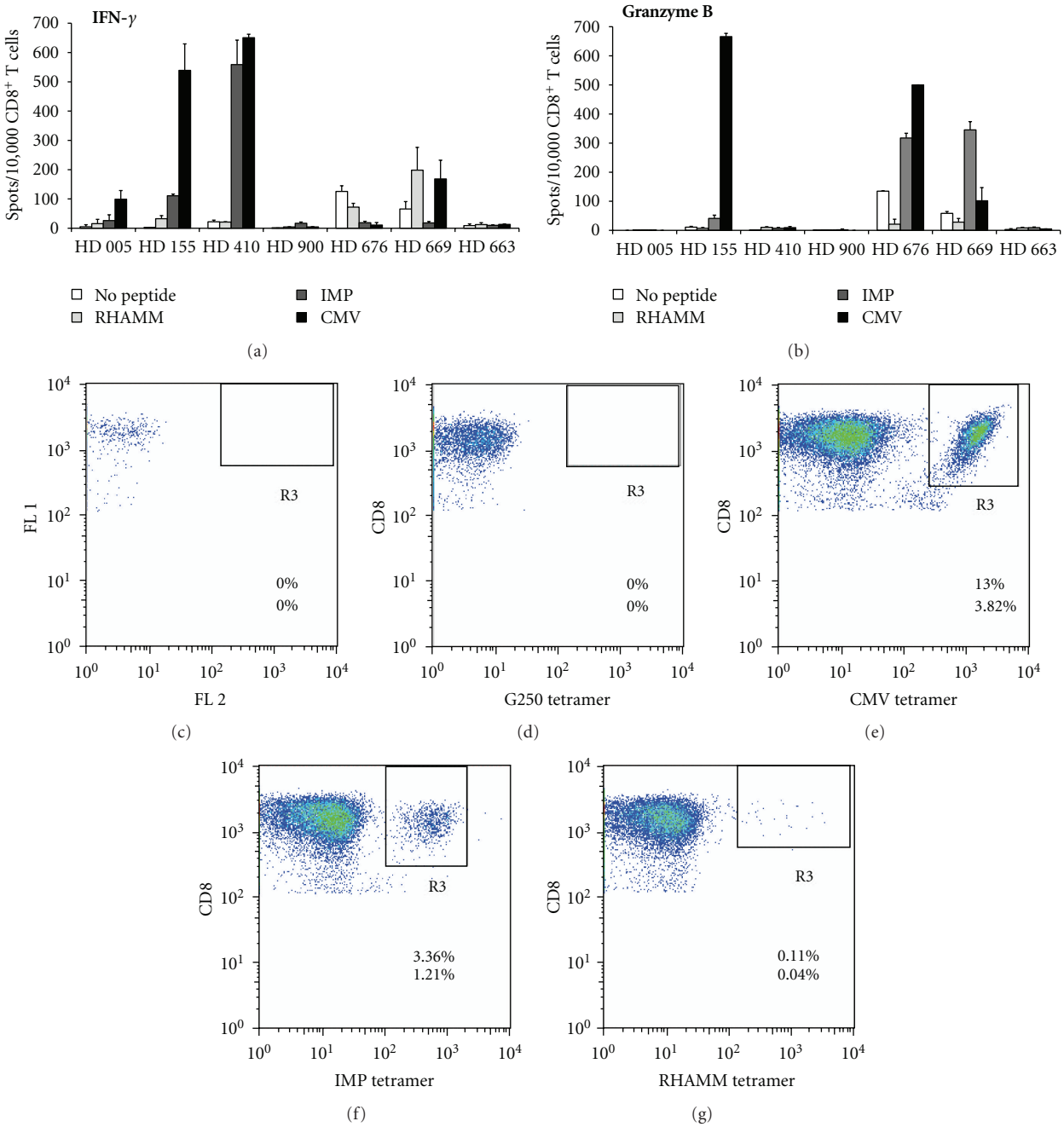

Figure 2: RHAMM-specific CD8$^+$ T-cell frequencies in healthy donors. Cells were stimulated in an MLPC for 7 days with different peptides and tested for their reactivity in ELISPOT assays for IFN-γ (a) and granzyme B (b) release. Stimulation of cells without any peptide was used as negative control (no peptide), whereas T cells stimulated with CMV and IMP peptides served as positive controls. RHAMM-specific T cells could be detected in two healthy donors (HD 155 and HD 669) by IFN-γ ELISPOT, (c)–(g) RHAMM-specific T-cell frequencies were determined by flow cytometry in HD 155. Reported frequencies correspond to gated CD3$^+$CD8$^+$ T cells (upper numbers), and from all cells in the lymphocyte gate (lower numbers). (c) Fluorescence minus one (FMO) was used as negative control to assess the intrinsic fluorescence of the cells. (d) As a further negative control, cells were cultured in the absence of any peptide and stained with tetramers specific for the irrelevant antigen G250. As positive controls, CD8$^+$ T cells were stimulated with either (e) CMVpp65 peptide or (f) IMP-derived peptide, (g) CD8$^+$ T cells were stimulated with RHAMM peptide, (e)–(g) CD8$^+$ T cells were stained with respective tetramers.

In another AML patient (Figure 4), RHAMM-specific CD8$^+$ T cells were detected by flow cytometry which certainly constituted a distinct subpopulation of RHAMM-specific CD8$^+$ T cells (Figures 4(f) and 4(j)), as proven by a number of negative controls (Figures 4(c)–4(e) and 4(g)–4(i)) including RHAMM-tetramer stained CD8$^+$ cells which were not stimulated by any peptide (Figures 4(e) and 4(i)). Interestingly we observed a general activation of CD8$^+$ T cells

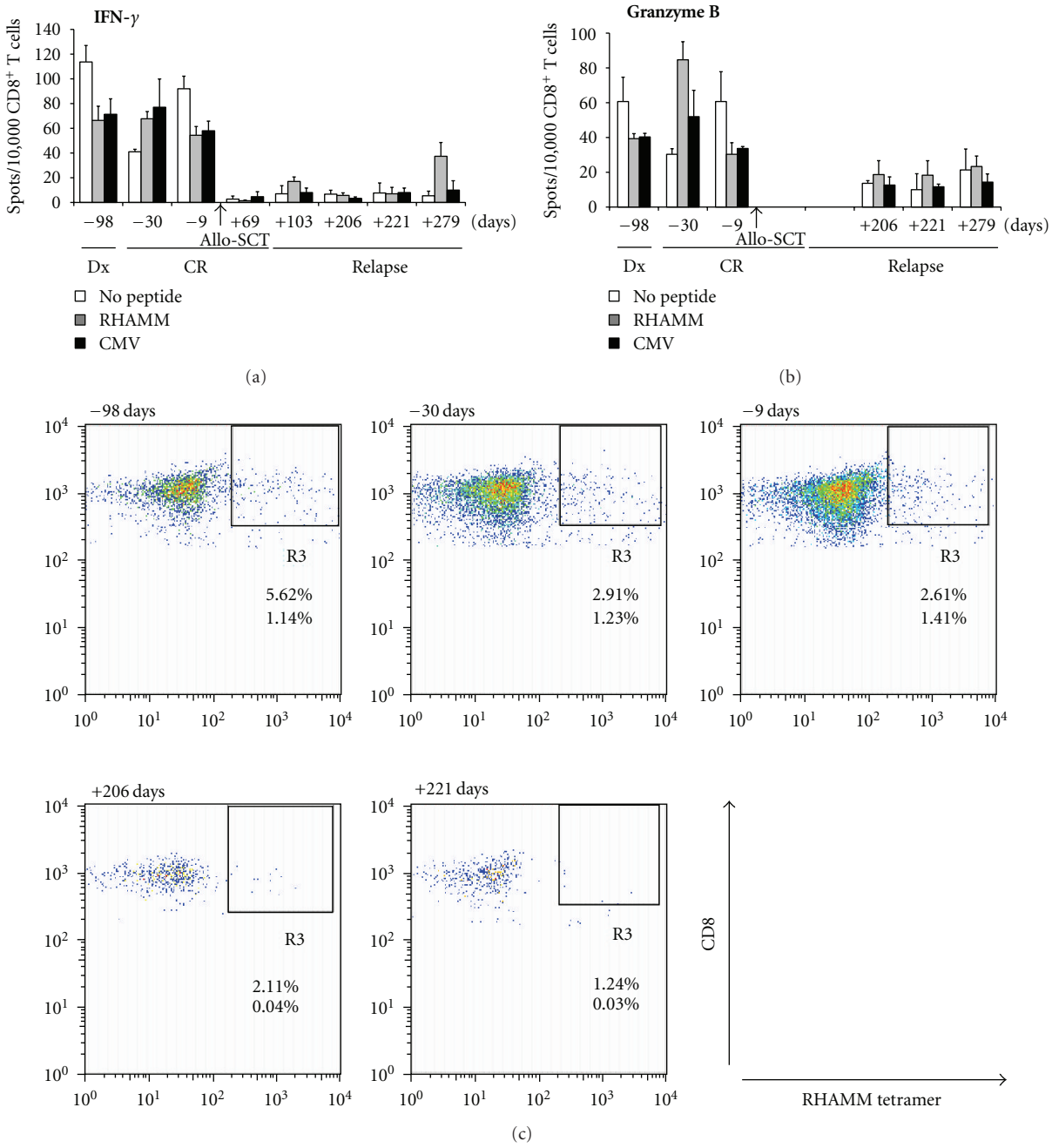

Figure 3: Longitudinal study of RHAMM-specific CTLs in a patient with AML that received allo-SCT. PBMCs were collected from an AML patient at different time points before or after allo-SCT as indicated and subjected to MLPC. $CD8^+$ T cells showed RHAMM-specific release of IFN-γ (a) and granzyme B (b) at one time point (30 days prior to transplant) when the patient was in CR. This active T-cell population was lost after allo-SCT and not reconstituted when the patient relapsed. (c) $CD8^+$ T cells were stimulated with RHAMM peptide and stained with respective tetramer. Frequencies of RHAMM-specific CTLs vanished over the time, as detected by flow cytometry. Reported frequencies correspond to the gate of $CD3^+CD8^+$ T cells (upper numbers) and to all cells in the lymphocyte gate (lower numbers).

at the time of relapse of the patient with no difference of smoldering and progressive disease(Figures 4(a) and 4(b)). This might be due to high concentrations of RHAMM on proliferating malignant cells stimulating specific T-cell responses.

In a third patient with AML antigen-specific $CD8^+$ T cells were detected in both peripheral blood (PB) and bone marrow (BM) to release IFN-γ and granzyme B in ELISPOT assays (Figure 5). RHAMM-specific $CD8^+$ T cells were able to release IFN-γ and granzyme B when the patient relapsed

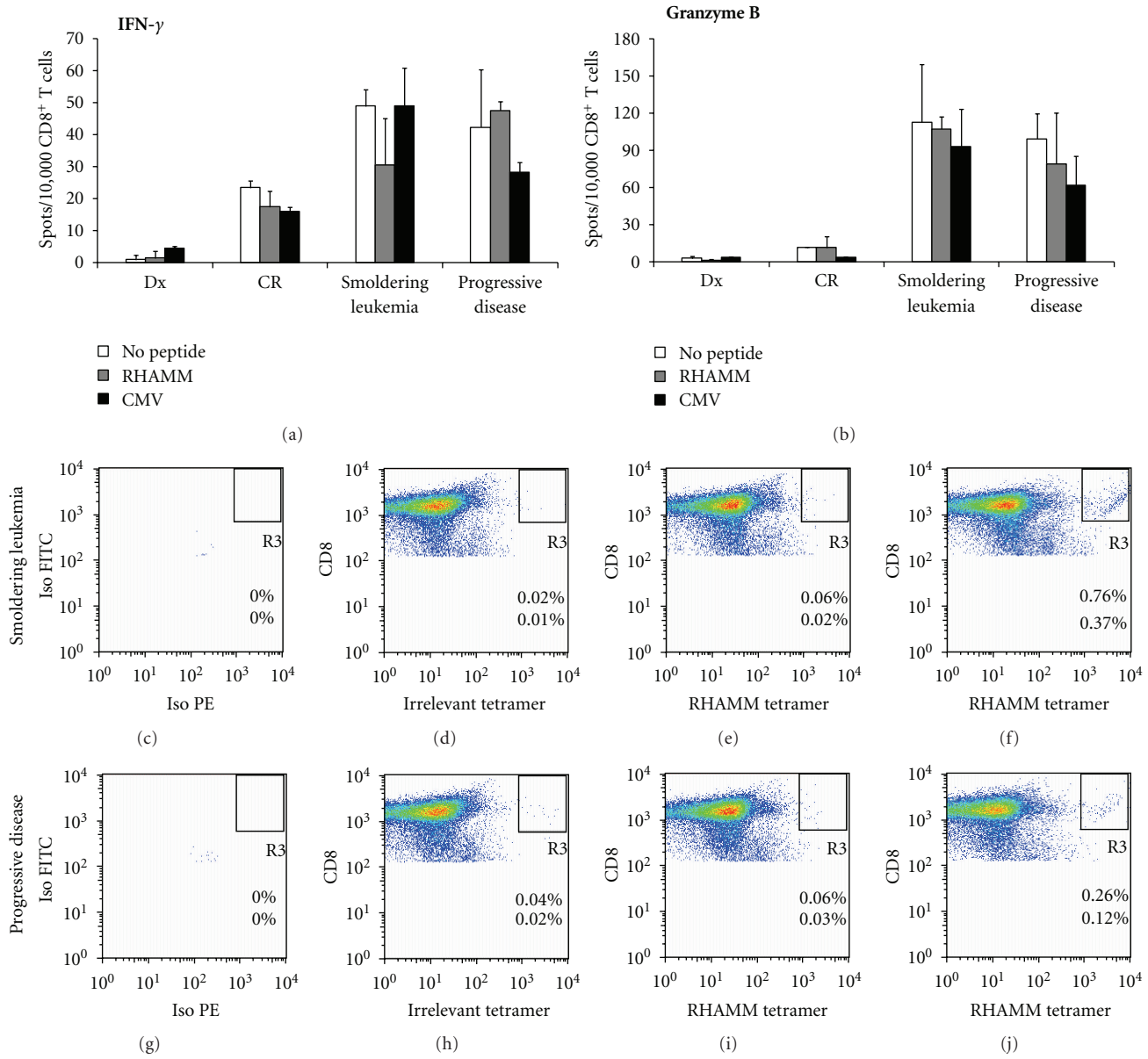

Figure 4: RHAMM-specific CTLs are present, but not functional. PBMCs were obtained from an AML patient who received chemotherapy. (a), (b). RHAMM-specific CTLs detected by flow cytometry at the stage of smoldering leukemia (c)–(f) and at the stage of progressive disease (g)–(j) did not release neither IFN-γ (a), nor granzyme B (b) at a level higher than background (no peptide control) as assessed by ELISPOT assays. (c)–(j) Reported frequencies correspond to all cells in the $CD3^+CD8^+$ T-cell gate (upper numbers), and to all cells in the lymphocyte gate (lower numbers). (c), (g) Isotype negative control, (d), (h) Non-peptide negative control, stained with an irrelevant tetramer, (e), (i) Non-peptide negative control, stained with RHAMM tetramer, (f), (j) $CD8^+$ T cells were stimulated with RHAMM peptide and stained with RHAMM tetramer.

from the disease. This RHAMM-specific CTLs response vanished after therapy with the DNA-methyltransferase inhibitor, azacitidine, and particularly after allo-SCT following a conditioning regimen with fludarabine, amsacrine, and cytarabine (FLAMSA) [6].

4. Discussion

In this work we evaluated the expression of RHAMM in 48 patients suffering from AML/MDS. We investigated the expression of RHAMM at RNA level using RQ-RT-PCR in patients before and after treatment. Additionally, we assessed the frequency of specific CTLs for this antigen. These molecular and immunological parameters were correlated with the clinical status of the patients.

RHAMM was firstly described as a soluble binding protein [7]. It is involved in motility, adhesion, proliferation, migration, and angiogenesis [8–11]. RHAMM is also crucial for transformation, metastasis, invasion, growth, and modification of the RAS signaling cascade [12–16].

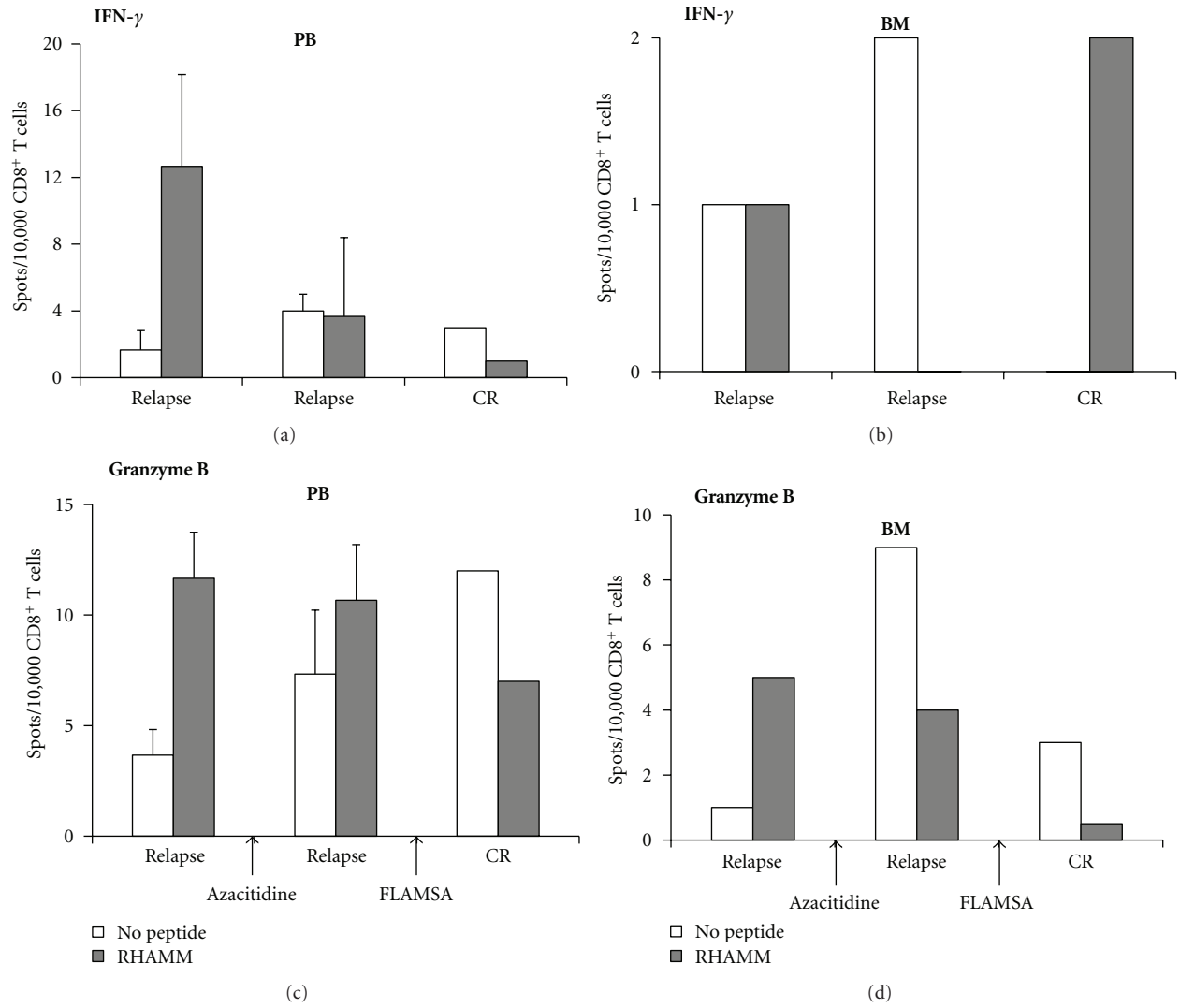

Figure 5: RHAMM-specific CTLs in a patient during relapse of AML. Release of both IFN-γ (upper panel) and granzyme B (lower panel) by RHAMM-specific CTLs was detected by ELISPOT in peripheral blood ((a), (b)) and bone marrow ((c), (d)) of a patient at the time of relapse. This population was lost following treatment with azacitidine and conditioning according to the FLAMSA protocol [6] followed by allo-SCT. Cells were not enough to perform flow cytometry analysis. Please note different y-axis scaling in this figure.

Multiple forms of RHAMM are overexpressed in a broad variety of solid tumors such as endometrial carcinoma [17], breast cancer [18], pancreatic cancer [19], stomach cancer [20], squamous cell lung carcinoma [21], and malignant melanoma [22], as well as in hematological malignancies like AML, MDS, B-CLL, and multiple myeloma [23, 24]. Seventy percent of AML patients overexpressed RHAMM at both mRNA and protein level [24, 25]. The overexpression of RHAMM mRNA and protein is associated with poor outcome and increased peripheral metastasis in breast cancer patients [26]. Recently, the expression of RHAMM has been reported to be a dismal prognostic factor in AML [27]. RHAMM was identified as one of the most promising LAAs in AML [2, 24]. The nonamer ILSLELMKL (designated R3), position 165–173, is the most immunogenic epitope [4] and it can be naturally processed and presented in an HLA-A2-restricted manner. RHAMM-R3 elicits both humoral and cellular responses in patients with leukemias but not in healthy donors or patients with autoimmune diseases [2, 24, 25]. Moreover, RHAMM-specific CTLs are able to lyse autologous RHAMM$^+$ blasts [4, 28]. Clinical vaccination of AML, MDS, MM, and CLL patients with the RHAMM-R3 peptide elicited specific immunological and hematological responses. Functionally active RHAMM-R3-specific CTLs were detected by tetramer staining in 70% of patients [2, 29, 30]. Peptide vaccination with RHAMM-R3 was safe and effective.

Here we aimed to determine the expression of RHAMM and the spontaneous presence of specific T cells reacting against this LAA in patients with AML/MDS before and after allo-SCT and/or conventional chemotherapy.

Little is known about the prognostic role of RHAMM and its interaction partners in leukemia. Tzankov et al. [27] analyzed RHAMM expression at the protein level on bone

marrow biopsies of a large cohort of AML patients. They found that 28% of the patients were RHAMM positive and that RHAMM could be a good prognostic factor at the protein level. However, no systematic study has been done to investigate the role of RHAMM as a prognostic factor at RNA level. To the best of our knowledge this is the first report using RQ-RT-PCR to measure *RHAMM* transcripts and to determine the immune T-cell response before and after allo-SCT. In the present study, we established a robust procedure to quantify absolute copy numbers of *RHAMM* using RQ-RT-PCR. The expression of *RHAMM* was not significantly different with respect to the *FLT3-ITD* status, karyotype, and gender of the patients. In the present study, 59% of the *de novo* AML patients expressed RHAMM. This finding is consistent with work by our group [31]. In our earlier series, 70% (35/50) of the AML patients were positive for *RHAMM* as determined by conventional RT-PCR [31]. Interestingly, some of the patients in the present study who were RHAMM negative at the time of diagnosis tested positive for RHAMM expression during clinical CR. This might be due to reconstitution of hematopoiesis as RHAMM might be also expressed early in stem cell cultures like the other LAA WT1 [32]. Another explanation might be that blasts in the bone marrow of the patient proliferated and therefore overexpressed RHAMM. This may also offer an explanation for the higher copy numbers of *RHAMM* in patients that received allo-SCT and reached CR compared to those treated under conventional chemotherapy.

We hypothesized that the presence of LAAs-specific T cells may be at least in part involved in the maintenance of the CR of patients. Functional RHAMM-specific CTLs were detected by ELISPOT in AML patients (Figures 3 and 5). Nevertheless, this population gradually vanished in the peripheral blood of patients after they received chemotherapy. Potentially downregulating effects of chemotherapy on T cells have been reported previously [6, 33, 34]. Interestingly the frequency of RHAMM-specific T cells increased in the bone marrow which might indicate a trafficking of these cells into the bone marrow [35].

In some of the ELISPOT assays we observed a general activation of CTLs (Figures 3(a), 3(b), 4(a), and 4(b)) which may be at least in part due to an inflammatory cytokine milieu caused by a viral infection.

In summary, *RHAMM* transcripts were indistinctly expressed before and after chemotherapy and allo-SCT. Nevertheless, a clear higher expression of *RHAMM* was observed in those patients at CR that were under allo-SCT. Furthermore, we were able to detect RHAMM-specific $CD8^+$ T-cell responses in both healthy donors and AML/MDS patients with overexpression of RHAMM. After chemotherapy and allo-SCT the RHAMM-specific $CD8^+$ T-cell subpopulation lost its property to secrete IFN-γ or granzyme B and eventually vanished. Therefore the stimulation of this subpopulation by RHAMM-R3 peptide vaccination or the adoptive transfer of RHAMM-specific $CD8^+$ T cells might reinstall the antileukemic effect after chemotherapy and allo-SCT.

Acknowledgments

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Review Article

Towards Curative Cancer Immunotherapy: Overcoming Posttherapy Tumor Escape

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The past decade has witnessed the evolvement of cancer immunotherapy as an increasingly effective therapeutic modality, evidenced by the approval of two immune-based products by the FDA, that is, the cancer vaccine Provenge (sipuleucel-T) for prostate cancer and the antagonist antibody against cytotoxic T-lymphocyte antigen-4 (CTLA-4) ipilimumab for advanced melanoma. In addition, the clinical evaluations of a variety of promising immunotherapy drugs are well under way. Benefiting from more efficacious immunotherapeutic agents and treatment strategies, a number of recent clinical studies have achieved unprecedented therapeutic outcomes in some patients with certain types of cancers. Despite these advances, however, the efficacy of most cancer immunotherapies currently under clinical development has been modest. A recurring scenario is that therapeutic maneuvers initially led to measurable antitumor immune responses in cancer patients but ultimately failed to improve patient outcomes. It is increasingly recognized that tumor cells can antagonize therapy-induced immune attacks through a variety of counterregulation mechanisms, which represent a fundamental barrier to the success of cancer immunotherapy. Herein we summarize the findings from some recent preclinical and clinical studies, focusing on how tumor cells advance their survival and expansion by hijacking therapy-induced immune effector mechanisms that would otherwise mediate their destruction.

1. Introduction

Numerous studies utilizing a variety of animal models have firmly established that the host immunity fundamentally affects cancer development and progression through a process termed cancer immunoediting [1]. The immunoediting process consists of three distinct phases: elimination (host immune cells act to destroy tumor cells), equilibrium (residual tumors persist but their outgrowth is held in check by host immunity), and escape (outgrowth of tumor cells with reduced immunogenicity and/or increased capacity to attenuate or subvert host immunity). Compatible with the cancer immunoediting hypothesis, there is mounting evidence that a natural, unmanipulated host immune system can detect and respond to a developing tumor. The host-tumor interactions proceed through the three immunoediting phases either independently or in sequence, and the composite result of the process determines the outcome of tumor rejection, dormancy, or progression. Therefore, the presence of clinically apparent tumors indicates a failed attempt to control tumor progression by the host immunity due to its ineffectiveness or acquired tolerance. Thus, the goal of cancer immunotherapy is to elicit an effective antitumor immunity by engendering productive immune responses and breaking tumor-induced immune tolerance. It has been proposed that the cancer immunoediting process also occurs in humans and in therapeutic settings when established tumors are confronted by the host immunity that has been subjected to therapeutic manipulations [2]. Accordingly, the net result of immunoediting after therapy could be either cure (complete tumor eradication), or prolonged remission (persistence of dormant residual tumors), or relapse (tumor escape and

progression). A multitude of cancer immunotherapy strategies have been developed with the goal to achieve the first two outcomes.

2. Recent Advances in Cancer Immunotherapy

A more comprehensive review on the advances in the field of cancer immunotherapy can be found elsewhere [3–5]. Here, we briefly summarize some recent progresses, with the intention to outline the therapeutic strategies and reagents that may unexpectedly elicit counterproductive effects under certain circumstances.

2.1. Cancer Vaccines. The premise of therapeutic cancer vaccine is that tumor-reactive T cells (including CD8+ and CD4+ T cells) can be induced and expanded in patients by exposing the host immune system to tumor-associated antigens (TAAs). Numerous vaccine approaches have been developed to deliver tumor antigens to patients, aiming to induce, activate, and amplify tumor-specific T cells. Tumor antigens can be delivered in the form of antigenic peptides, recombinant proteins, DNA or RNA constructs, recombinant microbial vectors, tumor cell lysates, and irradiated whole tumor cells. Tumor antigens are expected to be uptaken and presented by professional antigen-presenting cells (APCs), that is, dendritic cells (DCs), thereby activating tumor antigen-specific T cells. It is generally believed that the activation status of DCs critically influences the effectiveness of vaccines. In this regard, granulocyte macrophage colony-stimulating factor (GM-CSF) is widely used as a DC-activating adjuvant. Irradiated, autologous, whole tumor cells engineered to produce GM-CSF (GVAX) have been used to immunize patients with metastatic melanoma, pancreatic cancer, renal cell cancer, prostate cancer, and lung cancer [6–10]. GM-CSF-secreting allogeneic tumor vaccines have also been employed to treat multiple types of cancer [10–12]. Sipuleucel-T, the first patient-specific vaccine approved by the FDA, is formulated by incubating patient-derived peripheral mononuclear cells with a fusion protein consisting of GM-CSF and a tumor-derived differentiation antigen (prostatic acid phosphatase) [13]. Besides GM-CSF, other major vaccine adjuvants include bacilli Calmette-Guerin (BCG) and toll-like receptor (TLR) agonists, for example, poly-ICLC for TLR3, LPS, and synthetic TLR4 agonists, imiquimod for TLR7, and CpG for TLR9.

2.2. Adoptive Cell Therapy (ACT). ACT is a form of immunotherapy which involves the transfusion of large numbers of autologous or allogeneic, tumor-reactive lymphocytes to tumor-bearing hosts. The source of autologous tumor-reactive lymphocytes can come from lymphocytes infiltrating the tumor (TIL) or bone marrow (MIL), or peripheral blood mononuclear cells (PBMC). The specificity of the lymphocytes used for transfer could be either polyclonal (reactive to multiple undefined tumor antigens), or monoclonal (specific for a single defined tumor antigen). Unfractionated polyclonal TILs, after *ex vivo* expansion, have been used to treat patients with metastatic melanoma, in conjunction with systemic recombinant IL2 [14, 15]. ACT using tumor-specific T-cell clones relies on the ability to isolate and activate antigen-specific T cells from patients' specimens and then clonally expand these cells by reiterative antigenic stimulation. Although this strategy has generated encouraging results in melanoma clinical trials, its broad application has been hindered by the need of a series of individualized and cumbersome procedures required to obtain sufficient numbers of tumor-specific T cells. These limitations can be circumvented by the transfer of PBMC-derived lymphocytes that have been transduced to express T-cell receptors (TCRs) with the desired antigen specificity. ACT using lymphocytes bearing genetically engineered TCRs exhibited therapeutic efficacy in patients with metastatic melanoma as well as other forms of cancer [16]. A variation of this TCR engineering strategy is the generation of chimeric antigen receptors (CARs) which combine the antigen-binding properties of a monoclonal antibody (extracellular domain) and intracellular T-cell signal transduction domain consisting of CD3- zeta chain in conjunction with costimulatory endodomains such as CD28, OX40, or 4-1BB. Recent clinical trials conducted by different groups using lymphocytes bearing CD19-specific CARs reported encouraging clinical responses in patients with B-cell malignancy [17–19]. Of note, a preparative chemotherapy regimen is routinely used prior to ACT to induce transient lymphodepletion which facilitates the engraftment, expansion, and survival of the infused lymphocytes [20].

2.3. Therapeutic Monoclonal Antibodies and Immune-Modulating Antibodies. Monoclonal antibodies (mAbs) have proven to be valuable therapeutic agents for cancer treatment resulting in clinical responses and survival benefits in some patients. Currently, there are eight clinically approved therapeutic mAbs targeting five tumor-associated proteins, including CD20 (rituximab, Ibritumomab tiuxetan, and tositumomab), CD33 (gemtuzumab), CD52 (alemtuzumab), HER2/neu (trastuzumab), and EGFR (cetuximab, panitumumab). These mAbs can directly target malignant cells and exert antitumor effects by antagonizing oncogenic pathways and opsonizing tumor cells to trigger antibody-dependent cellular cytotoxicity or phagocytosis [21]. It has also been suggested that tumor-targeting mAbs may enhance tumor antigen uptake and presentation, thereby activating antitumor T-cell responses [22, 23]. In addition, mAb bevacizumab blocks tumor angiogenesis by inhibiting vascular endothelial growth factor-A (VEGF-A) expressed by host cells.

It is known that the host immune system has evolved to control the balance between immune activation and tolerance with a set of delicate intrinsic mechanisms involving the functions of costimulatory or coinhibitory molecules. These immunomodulating mechanisms, which normally enable the host immunity to respond to invading pathogens while maintaining homeostasis, often become dysregulated in the presence of active malignancy. In moving beyond antibodies that directly target and kill tumor cells, a different class of mAb has emerged as important and potent modulators for productive immune responses. These mAbs target the

costimulatory or coinhibitory receptors expressed on activated T cells, and their corresponding ligands on APCs or tumor cells. The rationale is to accentuate the stimulatory signals with agonist mAbs or disrupt the inhibitory signals with antagonist mAbs (checkpoint blockade) [24, 25]. The prototypical immunomodulating antibody is ipilimumab directed against CTLA-4, a checkpoint molecule that negatively regulates T-cell activation and function. Administration of ipilimumab, either alone or in combination with a peptide vaccine or chemotherapy, demonstrated long-term survival benefits in patients with metastatic melanoma in randomized phase III clinical trials [26, 27]. Programmed death 1 (PD-1) is another inhibitory receptor expressed on activated T cells. PD-1 interacts with its two ligands PD-L1 and PD-L2. PD-L1 is broadly expressed on APCs, nonimmune tissues, and tumor cells, and its expression correlates with an unfavorable prognosis in multiple types of cancer [28]. Sustained expression of PD-1 on tumor-reactive T cells is associated with a functionally exhausted phenotype [29–31]. Humanized anti-PD-1 and anti-PD-L1 antibodies have been developed and are currently under clinical evaluations. Phase I trials conducted in patients with several types of solid tumors demonstrated that PD-1 blockade was well tolerated and can achieve objective responses in some patients [32]. Besides CTLA-4 and PD-1, T-cell immunoglobulin mucin-3 (Tim-3) and lymphocyte-activation gene-3 (LAG-3) appear to be potential targets for antibody blockade, based on accumulating evidence that both LAG-3 and Tim-3 synergize with PD-1 to attenuate antitumor T-cell responses [33–35].

Parallel to the development of antagonist antibodies against checkpoint proteins, monoclonal antibodies acting as agonists of stimulatory receptors have also been generated for the purpose of augmenting antitumor immune responses. These antibodies mainly target a group of TNF family costimulatory receptors, including CD40, CD134 (OX40), CD137 (4-1BB), and glucocorticoid-induced tumor necrosis factor receptor (GITR). Among these antibodies, agonist anti-CD40 Abs have been extensively studied and exhibited clinical activities in a range of tumor types [36–38].

2.4. Combination Therapy. The microenvironment of a growing tumor is rendered profoundly immunosuppressive by a variety of mechanisms [39]. The well-characterized mechanisms include immunosuppression mediated by regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), metabolizing enzyme indoleamine 2,3-dioxygenase (IDO), inhibitory molecule PD-L1, and immunosuppressive soluble factors (such as IL-10, TGFβ, prostaglandin E2, and VEGF). $CD4^+Foxp3^+$ Treg cells can negatively modulate DC functions and suppress the effector activities of helper CD4+ T cells (Th), cytotoxic CD8+ T cells (CTL), and natural killer cells (NK). The mechanisms of Treg-mediated suppression are not entirely clear, but may involve inhibitory surface molecules CTLA-4 and PD-1, immunosuppressive soluble factors TGFβ, IL-10, and IL-35, and cytolytic molecules granzyme B and perforin [40]. MDSC can suppress immune responses through the activities of arginase 1, inducible nitric oxide synthase (iNOS), nitric oxide (NO), and reactive oxygen species (ROS) [41]. IDO and the related enzyme Tryptophan-2,3-dioxygenase (TDO) catalyze the degradation of the essential amino acids tryptophan (Trp) into kynurenine (Kyn), resulting in immune tolerance by reducing the local concentration of Trp required by T cells, and by the direct immune-inhibiting effects of Kyn [42]. In addition, Kyn has recently been identified as a natural ligand for the aryl hydrocarbon receptor (AHR) [43], which is involved in tumorigenesis, inflammation, and Treg development [44, 45]. It has been shown that IDO-expressing DCs can activate Tregs, which in turn mediate suppression through the PD-1/PD-L1 pathway [46]. These immunosuppressive mechanisms may operate simultaneously or sequentially, forming a mutually compensatory and self-reinforcing tolerogenic network. Conceivably, any form of immunotherapy has to overcome these hurdles to be effective. At present, the consensus in the field is that a combinatorial strategy has to be taken to target multiple immune pathways to attain durable antitumor effects.

Various forms of immunotherapy have been used in combination with conventional chemotherapy. Notably, most adoptive cell therapy protocols contain a lymphodepleting chemotherapy regimen in which the alkylating agent cyclophosphamide (CTX) is a major component. The immunoenhancing effects of CTX include "creating space" (i.e., providing access to limiting concentrations of cytokines and survival factors), depleting/inactivating Tregs [47], inducing the release of multiple growth factors and proinflammatory cytokines/chemokines [48, 49], all of which promote donor cell activation, expansion, survival, and memory formation [50, 51]. In terms of cancer vaccines, it has been well documented that chemotherapy can enhance the efficacy of GM-CSF whole-cell vaccines in both humans and mice [52, 53]. For immunomodulating antibodies, the combined use of chemotherapy with anti-CD40 agonist mAb [38], or anti-CTLA-4 antagonist mAb ipilimumab [27], both generated objective responses in patients.

A large body of preclinical work has shown enhanced therapeutic efficacy by combining different forms of immunotherapy. Administration of adjuvant IL-7 can enhance vaccine-induced antitumor immune responses by fostering a proinflammatory milieu and antagonizing Treg suppression and TGFβ signaling [54]. IFNα has been shown to enhance GM-CSF vaccine and peptide vaccine [55, 56], likely due to its immunostimulatory effects on DCs and T cells but inhibitory effects on Tregs [57, 58]. Conjugation of IFNα with a therapeutic antibody targeting CD20 seemed to increase tumor cytotoxicity while reducing the side effects associated with IFNα [59, 60]. In a mouse model of metastatic renal cancer, the use of anti-CD40 agonist mAb in combination with IL-2 or IL-15 can mediate tumor regression by inducing an inflammatory milieu that inhibited intratumoral Treg and MDSC activities [61]. The combined use of antibody blockade targeting different checkpoint molecules, such as anti-CTLA-4 and anti-PD-1 mAbs, anti-PD-1 mAbs, and anti-Tim-3 or anti-Lag-3, exhibited additive or synergistic effects in preclinical models [33, 35, 62], prompting the design of parallel clinical studies.

In summary, the growing availability of novel immunotherapeutic reagents offers immense options for combination therapy. These and many other potentially synergistic treatment combinations are areas of active investigation. Importantly, whereas combination therapies were historically developed empirically, with only complementary dose limiting toxicities as a driving principle, mechanistic studies of discrete elements of the host immune response provide a rationale for specific combinations, the choice of which may ultimately be tailored to individual patients ("personalized health care").

3. Immunological Features Associated with Effective Therapies

The onset, magnitude, and duration of the elicited immune responses vary dramatically from different forms of immunotherapies and vary from different individuals undergoing the same therapy. Currently, there remains a knowledge gap between the observed variations in therapy-elicited immune responses and the ultimate treatment outcomes, which may range from durable remission, disease stabilization, to initial remission followed by relapse, and exacerbated tumor progression. Here, we attempt to summarize the common features of the immune responses elicited by effective therapies—those that have led to beneficial outcomes in clinical studies or demonstrated efficacy in preclinical models. These features may shed light on the mechanisms of tumor immune escape in some cases, whereby an initial robust antitumor immunity elicited by therapy is subsequently rendered tolerant and unproductive.

3.1. Therapy-Induced Acute Inflammation. It has become increasingly clear that inflammatory responses play critical roles at different stages of tumor development, including initiation, progression, and metastasis [63]. These inflammatory responses are usually chronic in nature and inherent to many types of cancer, especially solid malignancies. Nonetheless, inflammation can also be induced by various cancer therapies, although it remains controversial whether therapy-induced inflammation is beneficial or detrimental to the hosts [64, 65].

It is not unexpected that many cancer immunotherapies, especially those involving chemotherapy, cause inflammation in treated hosts. Chemotherapeutic agents such as doxorubicin and oxaliplatin can cause massive tumor cell death and tissue damages, releasing various danger signals, such as high-mobility group protein B1 (HMGB1), calreticulin (CRT), and adenosine triphosphate (ATP) [66–68]. These danger/stress signals activate the innate immune system, resulting in rapid production of proinflammatory cytokines such as IL-1β and IL-17, leading to the activation of the adaptive immune system [67, 69]. Another well-studied chemotherapeutic agent is cyclophosphamide (CTX), which has been shown to rapidly induce the release of growth factors (including IL-2, IL-7, IL-15, and GM-CSF), proinflammatory cytokines (including IFNγ, IFNα/β, IL-1β, IL-6, and IL-17), and chemokines in treated tumor-bearing hosts [48, 49].

In some cases, inflammation can be induced by tumor-reactive T cells. This is exemplified by a recent clinical trial in which a patient with refractory chronic lymphocytic leukemia (CLL) received infusion of CD19-specific CAR-modified autologous T cells following chemotherapy preconditioning with CTX [18]. Complete remission was achieved 3 weeks after treatment, and the clinical response was accompanied by a delayed, temporal increase of inflammatory cytokines IFNγ and IL-6, and IFNγ-responsive chemokines CXCL-9 and CXCL-10, in blood and bone marrow. Since chemotherapy, like CTX used in this trial, usually induces a rapid and transient surge of inflammatory cytokines/chemokines that resolve in days [48], thus the delayed (15–30 days after T-cell infusion) emergence of inflammatory cytokines was likely the consequence of T-cell-mediated antitumor responses.

Moreover, immunotherapies without the involvement of chemotherapy can also induce inflammation. In an animal model in which vaccination was administered in conjunction with adjuvant IL-7, improved antitumor responses and survival were associated with increased serum concentration of IL-6, IL1α, IL-1β, IL-12, IL-17, TNFα, and chemokines CCL-5 and CCL-3 [54]. In a mouse model of renal cancer, the efficacy of IL-2/anti-CD40 agonist antibody was associated with conversion of the immunosuppressive tumor milieu to an immunogenic milieu which was rich of inflammatory chemokines including CXCL-9, CXCL-10, CCL-5, and CCL-3 [61]. Although the underlying mechanism of therapy-induced inflammation has not been defined, some recent studies suggest that tumor-reactive CD4+ T cells play an important role in initiating and modifying the inflammatory milieu [70–72].

Taken together, effective cancer therapies tend to generate an acute-type inflammation, which is typically associated with increased levels of IL-1, IL-6, IL-17, IFNγ, IFNα/β, and several IFNγ-responsive chemokines.

3.2. Mitigation of Local Immunosuppression. Since the discovery of the immunosuppressive properties of Treg cells, depletion or inactivation of Tregs has become an important component of many immunotherapeutic strategies. Low-dose CTX can effectively deplete Tregs and restore robust antitumor immune responses [47, 73]. Imatinib (Gleevec), a tyrosine kinase inhibitor used as targeted therapy for several types of cancers, was recently shown to promote Treg apoptosis [74]. Some immunotherapeutic agents blunt Treg-mediated suppression by mechanisms other than systemic depletion of Treg cells. Anti-CTLA-4 antagonist mAb (ipilimumab) does not reduce Treg cell numbers but instead preferentially exclude Tregs from the tumor lesion, so as to increase the intratumoral $T_{\text{effector}}/T_{\text{reg}}$ ratio and improve therapeutic efficacy [75, 76]. Anti-OX-40 agonist mAb has been shown to promote tumor rejection by inactivating Tregs [77–79]. Interestingly, coadministration of CTX and anti-OX-40 agonist mAb in a mouse melanoma model led to profound intratumoral Treg depletion accompanied by

an influx of effector T cells [80], suggesting that combined use of multiple Treg-targeting agents may be more effective.

4. Counterregulation Mechanisms That May Lead to Posttherapy Tumor Escape

It is not uncommon both in clinical studies and animal models that some cancer immunotherapies can induce measurable immune responses, but these responses did not translate into durable beneficial outcomes, suggesting the occurrence of tumor immune escape. Many factors may contribute to tumor escape under the immune pressure imposed by therapies, including immunogenicity alterations (downregulation of MHC expression, loss of antigen) in tumor cells in response to therapy, amplification of immunosuppressive cells (Tregs, MDSCs), and induction of immune checkpoint molecules in effector T cells (CTLA-4, PD-1). There is emerging evidence that certain components of the therapy regimen, and/or some immune products induced by therapy, may induce or exacerbate some of these tumor escape pathways. In this section, we discuss the possible mechanisms by which tumors antagonize and subvert robust immune responses in the posttherapy setting, thereby promoting tumor escape.

4.1. Treg Expansion or Repopulation after Therapy. Many cancer immunotherapies use vaccines to induce and amplify tumor-specific T cells. However, we initially reported that a vaccinia virus-based therapeutic vaccine indiscriminately expanded both CD4+ effector T cells and Tregs in tumor-bearing hosts, resulting compromised antitumor immune responses [81, 82]. This observation was further confirmed in various animal tumor models and clinical studies. In a spontaneous murine mammary tumor model, repeated IL-12/GM-CSF therapy led to a progressive increase of tumor-infiltrating Treg cells that impeded long-term antitumor effects [83]. In a melanoma animal model, LaCelle et al. reported that multiple rounds of GM-CSF whole-cell vaccinations during immune reconstitution of the lymphopenic hosts augmented the number of Treg cells and were associated with diminished antitumor potency of the T-cell repertoire [84]. In patients with HPV16-induced vulvar intraepithelial neoplasia, therapeutic vaccination with HPV16 E6/E7 synthetic long peptides led to increased frequencies of HPV16-specific Treg cells in a subset of patients, among which clinical responses were absent [85].

IL-2, a T-cell growth factor, has been approved by the FDA for the treatment of patients with metastatic melanoma and renal cell carcinoma. However, only 15% to 20% of treated patients experienced a clinical response, with 7% complete long-term responders [86]. The limited efficacy is likely in part due to the fact that IL-2 can significantly increase the frequency of functional Treg cells [86–88]. Indeed, daily injection of low dose of IL-2 has recently been reported to be successful in treating the immunopathology associated with *chronic* graft versus host disease in association with amplification of Treg numbers in allogeneic bone marrow transplant recipients [89].

TLR ligands have been frequently used as immune adjuvants to enhance antitumor immunity and are thought to be able to break immune tolerance by directly or indirectly inhibiting Treg responses [90–92]. However, it has been reported that some TLR agonists, including TLR4 ligand LPS and TLR5 ligand flagellin, can induce the proliferation and enhance the suppressive function of Treg cells [93, 94]. It is increasingly recognized that TLR agonists can generate suppressive as well as inflammatory responses in innate immune cells and can promote the induction of regulatory as well as effector T cells [95].

Currently available approaches that seek to abrogate Treg-mediated suppression, including cyclophosphamide, anti-OX-40 agonist mAb, and anti-CTLA-4 antagonist mAb, all seem to have limitations. Depletion of Treg cells by cyclophosphamide is transient and followed by rapid Treg repopulation, thus repeated administration of this drug may be required to stem Treg recovery [96, 97]. Recent studies suggest that anti-OX-40 agonist mAb may have dual functions on Tregs depending on environmental cues such as the local cytokine milieu. One study reported that OX40 signaling regulated Treg responsiveness to IL-2 and was required for sustaining Treg competitive fitness *in vivo* during repopulation of lymphopenic hosts [98]. Another report showed that anti-OX-40 agonist mAb blocked TGF-β1-mediated Treg conversion of activated T cells through enhanced production of Th1 or Th2 cytokines but promoted Treg proliferation and survival when IFNγ or IL-4 was absent in the local environment [99]. Anti-CTLA-4 antagonist mAb (ipilimumab), though proven to be effective in reducing the presence of Tregs within tumor, actually expands the overall number of Treg cells [75, 76, 80]. These features present the potential risk that Tregs may reestablish and repopulate in residual tumors and hence compromise long-term therapeutic effects.

Some therapeutic maneuvers, such as total body irradiation (TBI) followed by bone marrow transplantation in conjunction with infusion of immune lymphocytes, have also been shown to preferentially expand T-effector cells over Treg cells in a limited time window [100, 101]. However, host-derived radioresistant residual Tregs can rebound to occupy a niche in lymphopenic transplantation recipients [102], and functional Tregs de novo induced from the donor lymphocytes can repopulate in the hosts to reestablish immune tolerance [103, 104].

4.2. Expansion of MDSC. GM-CSF cancer vaccines can potently stimulate antitumor immune responses, in part, by causing the growth and differentiation of DCs [105]. However, it has been documented that chronic production or high-doses of GM-CSF can adversely affect antitumor immune responses by recruiting and expanding immunosuppressive MDSCs in animal tumor models [106–108]. Similar adverse effects of GM-CSF-based vaccines have been reported in some clinical studies [109–111].

In addition to GM-CSF, IL-1β, IL-6, and IL-17, inflammatory cytokines frequently induced after therapies can also drive MDSC expansion in tumor [112–114]. In fact,

many inflammatory cytokines have dual immunoregulatory activities, which could either enhance or attenuate tumor immunity. For example, on one hand, IL-1β can promote helper T-cell differentiation and inhibit Treg suppression [115–117]. On the other hand, IL-1β can promote tumor angiogenesis and metastasis [118, 119]. Thus, a balanced inflammatory milieu after therapy may be critical for durable antitumor effects [120]. In the case of IL-1β, unbalanced levels of IL-1β, either too much (MDSCs preferentially expand) or too little (Tregs become prevalent), all lead to tumor progression [114].

4.3. Survival and Proliferation of Residual Tumors. The use of chemotherapeutic agents has become an integral component of many cancer immunotherapies. In addition to debulking of the tumor mass, some anticancer drugs benefit cancer therapy by potentiating host antitumor immunity [121, 122]. However, chemotherapy almost invariably has certain side effects. While most of the side effects are associated with the global cytotoxicity of anticancer drugs, there is emerging evidence that some agents may even exert "opposite effects" that can enhance the malignancy of the treated cancers. For example, it has been shown previously that under certain experimental settings, cyclophosphamide treatment may render mice more prone to tumor metastasis by facilitating tumor cell intravascular proliferation, extravasation, and colony formation [123]. A recent study showed that chemotherapy with cisplatin or paclitaxel can induce VEGFR-1 expression on endothelial cells, creating an environment favorable to tumor cell retention and metastasis [124]. A different study reported that conditioned medium from BMDCs and plasma from paclitaxel-treated mice can promote metastatic properties in tumor cells *in vitro* by inducing matrix metalloproteinase-9 (MMP9), and paclitaxel treatment of mice with Lewis lung carcinoma led to accelerated MMP9-dependent metastases [125]. These findings suggest that some chemotherapeutic agents can induce a cascade of host events that may potentially support the growth and spread of residual tumors. Furthermore, some inflammatory cytokines induced after chemotherapy may contribute to tumor relapse and chemoresistance [126]. In a mouse model of Burkitt's lymphoma, Gilbert and Hemann showed that IL-6 was released in the thymus in response to doxorubicin treatment, creating a "chemoresistant niche" that promotes the survival of a minimal residual tumor burden and serves as a reservoir for eventual tumor relapse [127]. Taken together, these studies illustrate that the prosurvival and prometastasis effects of some chemotherapeutic agents may confound the treatment outcomes of some cancer immunotherapies.

4.4. Upregulation of Checkpoint Molecules. It is well known that PD-L1 expression can be upregulated by both type I (IFNα/β) and type II (IFNγ) interferons, which are often induced after bacteria or viral infections, serving as a hard-wired counterregulation mechanism to avoid excessive immune responses [28, 128]. However, this mechanism also can be employed by residual tumors to counteract antitumor immunity elicited by therapy, which is often associated with the production of interferons. In addition to regulating PD-L1 expression, a recent study reported that IFNα can also augment PD-1 expression on antigen-stimulated T cells, rendering these T cells susceptible to PD-L1-mediated suppression [129]. Besides interferons, several common gamma-chain cytokines IL-2, IL-7, and IL-15, which are often elevated after chemotherapy, have been found to upregulate PD-1 as well as PD-L1 on T cells [130], suggesting similar mode of immune regulation.

It has been shown that PD-L1 plays an essential role in the development, maintenance, and function of Treg cells [131, 132]. Therefore, one of the anticipated benefits of PD-L1 blockade is to mitigate Treg-mediated suppression. However, a recent study presented evidence that PD-L1 blockade can expand $ICOS^+Foxp3^+$ $CD4^+$ regulatory T cells, which act to inhibit the optimal functions of CTLs [133]. This result is consistent with the notion that PD-L1 may negatively regulate Treg cells under certain circumstances, as in the case of chronic infection by hepatitis C virus [134]. These data provide a cautionary note for the possible opposing effects of PD-L1 blockade on tumor immunity.

4.5. Induction of IDO. Many immune adjuvants used in cancer immunotherapies, including lipopolysaccharide (TLR4 ligands), resiquimod (TLR7/8 ligands), CpG (TLR9 ligands), and anti-CD40 antibody, can induce IDO expression [135]. In addition, proinflammatory cytokines IFNγ and IFNα/β are potent IDO inducers [136]. Given IDO's broad activities in mediating direct T-cell suppression and Treg activation [46], posttherapy IDO induction represents a constant threat to long-term therapeutic efficacy. Thus, durable tumor remission may not be achievable unless induced IDO activity is blocked concomitantly. Supporting this notion, it has been shown in various model systems that inhibition of IDO by a clinically applicable inhibitor 1-methyl-tryptophan (1MT) can markedly improve the efficacy of a wide range of cancer therapies, including cytotoxic chemotherapy [137], IL-12/GM-CSF therapy [138], and targeted therapy [74].

5. Overcoming Tumor Escape with Combinatorial Treatment Strategy

The immune-tolerizing mechanisms discussed in the preceding section may also operate in tumor-bearing hosts prior to therapy. These mechanisms are subject to disruption by cancer immunotherapy, but under certain conditions, they can recover and reestablish immune tolerance to residual tumor cells. The reemergence of a tolerogenic mechanism can be driven by certain components of the therapy regimen, for instance, Treg expansion by IL-2 immunotherapy; alternatively, some therapy-induced immune mediators can counterregulate antitumor immune responses, for example, type I IFN can induce PD-1 in activated T cells and upregulate PD-L1 in tumor cells, and IL-1β and GM-CSF can recruit and activate MDSCs. It is unlikely that these tolerogenic mechanisms act in isolation; rather, they may be interactive and mutually compensatory. Therefore, an effective cancer immunotherapy requires a combinatorial

strategy to overcome tumor immune escape. With increased knowledge of tumor escape mechanism at work, rational combination of multiple targeted treatment approaches has shown tremendous potential in achieving curative outcomes in preclinical models. In a mouse renal cell carcinoma (RENCA) tumor model, Webster et al. showed that Tregs and PD-L1 collaborated to impair vaccine-induced recall response of tumor-specific memory T cells; correspondingly, treatment with tumor cell vaccines in combination with PD-L1 blockade and CD4+ T-cell depletion (triple therapy treatment) resulted in complete regression of large established tumors and raised durable protective immunity [139]. A recent study by Khleif's group reported that the combination of a tumor vaccine, PD-1 blockade, and CTX led to complete regression of established TC-1 tumors and improved survival benefits in a significant percentage of treated animals [140]. In this study, the combined use of PD-1 blockade and low-dose CTX markedly increased the number of vaccine-induced tumor-infiltrating CTLs while sustainably reducing systemic and local Tregs. In a mouse melanoma model, complete regression of advanced primary melanomas in the skin and metastases in the lung was achieved by a protocol which consisted of timed treatments of CTX preconditioning, adoptive transfer of tumor-specific CD8+ T cells, viral vector-based vaccinations, and administration of CpG-containing adjuvants [141]. These results provide compelling rationales for applying similar strategies in future clinical trials.

6. Conclusions

Current cancer immunotherapies rarely result in immediate and complete tumor eradication, and more often the residual tumors persist and likely form equilibrium with the host immunity, with the end result being either tumor recurrence or sustained remission. It is important to appreciate that during this process, components of the therapy regimens, such as various chemotherapeutic agents and immune adjuvants, and elements of therapy-induced responses, such as proinflammatory cytokines, can shift the balance one way or the other. The key challenge relies on synchronizing the immune-enhancing effects of all relevant factors while minimizing their collateral counterproductive effects. Our improved knowledge of the relations of tumor immunity and tumor counterregulation should help the design of more efficacious cancer immunotherapy strategies that lead to a curative outcome.

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Review Article

T Cells in Gastric Cancer: Friends or Foes

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Gastric cancer is the second cause of cancer-related deaths worldwide. *Helicobacter pylori* is the major risk factor for gastric cancer. As for any type of cancer, T cells are crucial for recognition and elimination of gastric tumor cells. Unfortunately T cells, instead of protecting from the onset of cancer, can contribute to oncogenesis. Herein we review the different types, "friend or foe", of T-cell response in gastric cancer.

1. Introduction

Gastric cancer (GC) is the second cause of cancer worldwide for cancer-related deaths [1]. The regional variations mainly reflect differences in the prevalence of *Helicobacter pylori* infection, which accounts for more than 60% of GC worldwide [2].

Helicobacter pylori infection is very common in human populations but only 1% of infected individuals develop gastric cancer in response to persistent infection [2–4]. Certainly *Helicobacter pylori* plays a crucial role [3–5], but also host factors are relevant for the outcome of the infection [6, 7]: actually, many studies showed that the subset of patients who progress to gastric cancer appear to have an increased incidence of some polymorphisms in proinflammatory cytokines and particularly IL-1β.

The response of the body to a cancer is not a unique mechanism but has many similarities with inflammation and wound healing. The last century Virchow's [8] observation of a close association between cancer and inflammation anticipated the current interest in the role of immunity in tumor pathogenesis.

Recent insights into the dynamics of the tumor microenvironment have begun to clarify the mechanisms underlying tumor-promoting inflammation, which bears striking similarities to wounds that fail to heal [9, 10]. Approximately 20% of cancer deaths worldwide are currently linked to unresolved infection or inflammation, with gastrointestinal malignancies representing a significant proportion of this disease burden: the most frequent associations are gastric carcinoma and *Helicobacter pylori* infection [3–10], colorectal carcinoma and inflammatory bowel disease [11], pancreatic carcinoma and chronic pancreatitis [12].

Unresolved inflammation generates a microenvironment that facilitates cellular transformation and the propagation of invasive disease. Chronic tissue damage triggers a repair response including the production of growth and survival factors, proangiogenesis cytokines, and immune regulatory networks [7, 8].

The release of inflammatory cell-derived reactive oxygen species coupled with stimulated epithelial cell proliferation creates an elevated risk of mutagenesis. In addition, crosstalk between neoplastic cells and immune elements throughout the smoldering inflammation perpetuates the transforming environment, which provides the evolving tumor cells with sufficient opportunity to acquire mutations and epigenetic alterations that are necessary for cell autonomy.

Inflammatory circuits can considerably differ in different tumors in terms of cellular and cytokine networks and

molecular drivers. However, macrophages are a common and fundamental component of cancer promoting inflammation. The drivers of macrophage functional orientation include tumor cells, cancer-associated fibroblasts, B cells, and T cells.

It is not unfrequent that gastric cancer patients with the same TNM stage pursue different clinical courses. Histopathologic classifications, including WHO classification [13] and molecular classifications [14], have also been applied for the prediction of patient survival, but their prognostic accuracies are controversial [13]. In addition, many attempts have been made to link molecular events in cancer cells with patient outcome, but none of these have been proved to be clinically meaningful. As a consequence, new prognostic determinants in conjunction with the TNM stage are required to more reliably and precisely predict patients' clinical course.

As the cancer immunosurveillance hypothesis was first proposed, the concept that the immune system can recognise and eliminate tumor cells has been energetically debated. Many experimental studies in rodents have shown that the immune system indeed functions to protect murine hosts against development of both chemically induced and spontaneous tumors [15]. Furthermore, in humans, epidemiologic investigations indicate that immunocompromised patients have a higher probability to develop cancers of both viral and nonviral origin, which supports the cancer immunosurveillance concept [15]. In addition, current evidence indicates a positive correlation between the presence of lymphocytes in tumor tissue and increased patient survival.

Recent studies have highlighted that several types of tumor infiltrating lymphocytes (TIL) are associated with a better disease outcome for various human cancers [16–18], demonstrating that higher numbers of $CD3^+$, $CD8^+$, or $CD45RO^+$ T cells in tumor tissue are significantly correlated with lower frequencies of lymph node metastasis, disease recurrence, or longer patient survival. Wang et al. advocated that the type, density, and location of immune cells in colorectal cancer have prognostic values that are superior to and independent of those of the TNM classification [16].

However, tumors have developed a number of different strategies to escape immune surveillance, such as the loss of tumor antigen expression, the expression of Fas ligand (Fas-L) or CD200 that can induce apoptosis in activated T cells, the secretion of immunosuppressive cytokines, such as IL-10 or TGF-β, or the generation of regulatory T cells, and MHC downregulation or loss [19]. An alteration in HLA class I expression occurs in many cancers, such as gastric cancer [20] and potentially plays a role in the clinical course of the disease by enabling tumor cells to escape T-cell-mediated immune responses [21]. Recent observations suggest that the induction of T-cell apoptosis coexisting with a downregulation of TCR-ζ molecules may be responsible for T-cell dysfunction in patients with gastric cancer [22].

Within TIL population, there are also T regulatory cells (Tregs), which are able to inhibit the immune response mediated by $CD4^+$ and $CD8^+$ T cells in preventing allograft rejection, graft *versus* host disease, and autoimmune disease [23, 24].

In cancer individuals, Tregs were found to downregulate the activity of effector function against tumors, resulting in T-cell dysfunction in cancer-bearing hosts [25, 26]. High numbers of Tregs were indeed reported in patients with different type of cancer [27–29] such as gastric and esophageal cancer [30]. These observations led us to the hypothesis that tumor-bearing hosts with advanced cancers have an increased population of Tregs, which might inhibit the tumor-specific T-cell response.

The aim of this paper is to highlight the role of different T-cell populations involved in gastric cancer immune response and to evaluate their impact in blocking/promoting the development of gastric cancer.

2. Protective Role of Cytotoxic T Cells

A large body of evidence indicates that in gastrointestinal malignancies, endogenous responses may inhibit tumor growth and perhaps modulate the clinical course of the disease. Many reports have been obtained on colorectal carcinoma: the type, density, and intratumoral location of the lymphocyte infiltrate have been shown to be more informative biomarkers than the TNM or Duke's classification [16]. In this context, dense infiltrates composed of cytotoxic memory T cells are strongly associated with a reduced risk of recurrence after surgery and increased overall survival. In particular, patients with early-stage cancers but an absence of T-cell infiltrates display poor outcomes, whereas subjects with significant tumor burdens but robust T-cell infiltrates showed improved outcomes [23, 31].

The prognostic role of tumor-infiltrating immune cells in patients with gastric cancer is largely unknown. Only a few reports have been issued on the association between tumor infiltrating immune cells and the clinical outcome in GC: Ishigami et al. [32] reported that patients showing a high level of natural killer cell infiltration in tumor tissues have a better prognosis, and Maehara et al. [33] showed that a high density of dendritic cell infiltration is associated with the absence of lymph node metastasis. On the other hand, the group of Fukuda [34] found no significant difference in survival between patients with marked or slight TIL infiltration. However, they detected TILs by immunostaining in GC patients, classified cases into groups with marked or slight TIL infiltration, and did not determine TIL numbers.

T-cell-mediated adaptive immunity is considered to play a major role in antitumor immunity. In mouse models, it has been demonstrated that adaptive immunity prevents the development of tumors and inhibit tumor progression [35].

Accordingly, recent data [36] showed that in GC high densities of immune cells related to adaptive immunity (especially cytotoxic T cells and memory T cells) are associated with favorable survival and indicate that adaptive immunity plays a role in the prevention of tumor progression.

TIL density is also correlated with the presence of lymph node metastasis but not with the depth of tumor invasion. On the basis of this finding, the authors suspect that the prognostic role of TIL is mainly due to decreased metastatic

potential and suggest the following possible mechanisms. First, the expansion of clones with metastatic potential usually containing larger amounts of aberrantly expressed proteins, including proteins that contribute to metastasis, which may act as tumor-associated antigens. As a result, these clones are more likely to be destroyed by in situ immune reactions. Second, a high density of TIL means a healthy immune system, and therefore, immune reaction occurring in lymph node may also exert a proper function against tumor cells that have drained into lymph nodes in patients with high TIL densities. Third, tumor burden of metastatic foci in lymph node is less bulky than those of primary foci, and thus, metastatic foci are more likely to be susceptible to complete destruction by immune reaction.

Many experimental and clinical observations suggest that metastatic growth in mice and humans is more difficult to control through vaccination and T-cell response, and new observations indicate that immunity against early and even preneoplastic lesions is stronger than against advanced tumors [37–39]. In some cases, enhanced anticancer T-cell activity may thus prevent metastasis rather than eliminating established metastatic nodules. Recently, Kim and coll. [40] evaluated the antitumor activity of *ex vivo* expanded T cells against human GC. 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%): 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 GC cells. 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 gastric cancer patients.

The adoptive immunotherapy of GC with CIK cells has been also reported in preclinical and clinical studies [41]. MHC-I-restricted CTLs from GC patients recognize tumor-associated antigen and react specifically against self-tumor cells [41–43]. One tumor-specific antigen, MG7-antigen, showed great potential for predicting early cancer as well as for inducing immune responses to GC [44, 45]. Using HLA-A-matched allogeneic GC cells to induce tumor-specific CTLs appears to be an alternative immunotherapy option for gastric cancer [38]. Also, CIK cells in combination with chemotherapy showed benefits for patients who suffer from advanced gastric cancers [46, 47]. The serum levels of tumor markers were significantly decreased, the host immune function was increased and the short-term curative effect as well as the quality of life, were improved in patients treated by chemotherapy plus CIK cells compared to those in patients treated by chemotherapy alone [48].

Most studies analyzing T-cell response to tumor-associated antigens (TAAs) have emphasized $CD8^+$ T cells thus far. However, $CD4^+$ T cells may play a crucial role in both the induction and activation of TAA-specific memory $CD8^+$ T cells toward cytotoxic effector T cells [49, 50].

Recently Amedei et al. [51] analyzed the functional properties of the T-cell response to different antigen peptides related to GC in patients with gastric adenocarcinoma. A T-cell response specific to different peptides of GC antigens tested was documented in 17 out of 20 patients. Most of the cancer peptide-specific TILs expressed a T helper 1 (Th1)/T cytotoxic 1 (Tc1) 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/Tc1 functional profile.

In conclusion, in most 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.

3. T Regulatory Cells in Cancer

The physiological role of Tregs is the protection against the autoimmune diseases through the direct suppression of T effector cells reacting against "self," although they can be also involved in the control of immune response against exogenous antigens [52]. Since most antigens expressed by neoplastic cells are "self"-antigens [53], it is commonly considered that Tregs are also involved in the suppression of the immune response against tumors, favoring tumor escape from immune response [54]. TILs consist of various antitumor effector and regulatory subsets. T-cell infiltration is associated with good tumor prognosis in many types of cancers. $CD8^+$ and $CD4^+$ T lymphocytes are effector cells thought to be associated with a favorable prognosis [55]. While $CD8^+$ T cells are the main effectors of antitumor immunity, $CD4^+$ T cells induce and maintain CD8 response [56]. On the other hand, regulatory lymphocytes, a subset of T cells which inhibit the antitumor immune reaction have been described to be associated with unfavorable prognosis [57–62]. Tregs cells are known to attenuate host antitumor immunity by suppressing T-cell proliferation, antigen presentation, and cytokine production [24]. As the tumor progresses and becomes established in the host, the population of TILs is skewed to favor regulatory T cells over the helper $CD4^+$ T cells [56].

Studies of regulatory T cells in GC are very few and have yielded conflicting results. Haas et al. [63] reported that stromal but not intraepithelial regulatory T cells are associated with a favorable prognosis. Mizukami et al. [64] reported that the localization pattern but not the absolute number of regulatory T cells was associated with the prognosis. In breast cancer, it has been demonstrated that pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating $Foxp3^+$ regulatory T cells [65]. It has been demonstrated that in some kidney tumors Treg frequency is significantly higher in patients with worse prognosis [66]. Conversely, several recent reports highlighted a protective role of Treg in cancer [67]. In renal cancer, Siddiqui et

al. showed no correlation between tumor-infiltrating Treg frequency and disease progression [67]. The significance of regulatory T cell in GC as a poor prognostic factor has also been investigated. Perrone et al. [62] and Shen et al. [68] reported unfavorable prognosis with increased intratumoral regulatory T cells.

Two different studies [69, 70] confirmed that GC cell can induce Tregs development via TGF-β1 production; in particular the level of serum TGF-β1 in GC patients (15.1 ± 5.5 ng/mL) was significantly higher than that of the gender- and age-matched healthy controls (10.3 ± 3.4 ng/mL). Furthermore, the higher TGF-β1 level correlated with the increased population of $CD4^+Foxp3^+$ Tregs in advanced GC. A significant higher frequency of $CD4^+Foxp3^+$ Tregs was observed in PBMCs cultured with the supernatant of MGC than GES-1 (10.6% ± 0.6% *versus* 8.7% ± 0.7%). Moreover, using the purified $CD4^+CD25^-$ T cells, the authors confirmed that the increased Tregs were mainly induced from the conversation of $CD4^+CD25^-$ naive T cells, and induced Tregs were functional and able to suppress the proliferation of effector T cells. Finally, they demonstrated that GC cells induced the increase in $CD4^+Foxp3^+$ Tregs via TGF-β1 production. Gastric cancer cells upregulated the production of TGF-β1 and the blockade of TGF-β1 partly abrogated Tregs phenotype.

The second study [70] investigated the frequency of $Foxp3^+$ Tregs within $CD4^+$ cells in TILs, regional lymph nodes, and PBL of GC patients. Furthermore, to elucidate the mechanisms behind Treg accumulation within tumors, authors evaluated the relationship between CCL17 or CCL22 expression and the frequency of $Foxp3^+$ Tregs in GC. $CD4^+CD25^+Foxp3^+$ Tregs were counted by flow cytometry and evaluated by immunohistochemistry. Moreover, an *in vitro* migration assay using Tregs derived from GC was performed in the presence of CCL17 or CCL22. As a result, the frequency of $Foxp3^+$ Tregs in TILs was significantly higher than that in normal gastric mucosa (12.4% ± 7.5% *versus* 4.1% ± 5.3%). Importantly, the increase in Tregs in TILs occurred to the same extent in early and advanced disease. Furthermore, the frequency of $CCL17^+$ or $CCL22^+$ cells among $CD14^+$ cells within tumors was significantly higher than that of normal gastric mucosa, and there was a significant correlation between the frequency of $CCL171^+$ or $CCL22^+$ cells and $Foxp3^+$ Tregs in TILs. In addition, the *in vitro* migration assay indicated that Tregs were significantly induced to migrate by CCL17 or CCL22. In conclusion, CCL17 and CCL22 within the tumor are related to the increased population of $Foxp3^+$ Tregs, with such an observation occurring in early GC.

Since the Tregs may restrain the antitumor activity of cytotoxic T cells, the balance of effector and suppressor cells may also prove to be a decisive factor in patient outcome, and a recent study [30] contains the first evidence related to the prevalence of Tregs in gastric and esophageal cancer. The authors shown increased populations of $CD4^+/CD25^+$cells in peripheral blood T cells from patients with gastric and esophageal cancers in comparison with healthy donors. Moreover, the population of $CD4^+/CD25^+$ cells in the TILs of GC was higher than that in normal gastric mucosa. Authors also confirmed that $CD4^+/CD25^+$ isolated from patient peripheral blood had a regulatory function by evaluating cytokine production and suppressive activity.

Moreover, the population of $CD4^+/CD25^+$ cells in the TILs of GC patients with advanced disease was significantly more extended than that in TILs of patients with early-stage disease or that in intraepithelial lymphocytes of normal gastric mucosa. As a functional consequence, $CD4^+/CD25^+$ cells did not produce IFN-γ but large amounts of IL-10. Also, the proliferation of $CD4^+/CD25^-$ cells was inhibited in the presence of $CD4^+/CD25^+$ cells in a dose-dependent manner, so confirming that $CD4^+/CD25^+$ has an inhibitory activity corresponding to Tregs.

Similar results were obtained by Shen and coll. [71], who demonstrated that increased CD4(+)CD25(+) CD127(low/−) regulatory T cells were also present in the tumor microenvironment, such as those found in the ascites fluid, tumor tissue, or adjacent lymph nodes. In addition, they found that CD4(+)CD25(+)CD127(low/−) Tregs suppressed effector T-cell proliferation and also correlated to advanced stage of GC, suggesting that CD4(+)CD25(+)CD127(low/−) can be used as a selective biomarker to enrich human Treg cells and also to perform functional *in vitro* assays in GC.

4. Th17 in Cancer

A new subset of Th cells, named Th17 cells, producing IL-17 alone or in combination with IFN-γ, has been identified [72]. Th17 cells may also secrete IL-6, IL-22, and TNF-α and play a critical role in protection against microbial challenges, particularly extracellular bacteria and fungi [73]. The role of Th17 cells in tumor immunology can be dichotomous: Th17 cells indeed seem to play a role both in tumorigenesis and eradication of an established tumor. Many laboratories have studied Th17 populations in blood and occasionally tissues of patients with various cancers. A potential protective effect of Th17 cells has been reported in cancer affecting mucosal tissues, such as gut, lung, and skin [74, 75]. An increase in Th17 cells has been detected in peripheral blood, tumor microenvironment and tumor-draining lymph nodes of several different human and mouse tumor types [76], such as ovarian cancer [77].

A recent study has shown that the number of Th17 cells increased in the TILs from melanoma, breast, and colon cancers [78]; Th17 cells were also suggested as a prognostic marker in hepatocellular carcinoma [79]. In contrast to data on solid tumors, little is known about Th17 cells in hematological malignancies. Serum IL-17 levels were recently shown to be elevated in patients with multiple myeloma, especially in stages II and III of the disease. Thus, current data confirm a role for IL-17 in the promotion of angiogenesis and in the progression of multiple myeloma [80]. Th17 cell frequencies and IL-17 concentrations were significantly higher in peripheral blood samples from untreated patients with acute myeloid leukemia than in those from healthy volunteers and were reduced in the former after chemotherapy [81].

On the other hand, some studies have found that the number of Th17 cells is decreased in several types of tumor. The levels of tumor-infiltrating Th17 cells and IL-17 in ascites were reduced in a group of ovarian cancer patients with more advanced disease and seemed to positively predict outcome [82]. A low number of Th17 cell is present in the tumor microenvironment of non-Hodgkin's lymphoma because malignant B cells may upregulate Treg cells and inhibit Th17 cells [83]. Th17 cells are present in much lower numbers in HER2-positive breast cancer patients than in either healthy controls or HER2-negative patients [84].

One study in prostate cancer demonstrated that Th17 cells infiltrating the tumor correlated inversely with the Gleason score [85]. This implied that Th17 cells mediate an antitumor effect in the development of prostate cancer. One group found that IL-17 promoted the tumorigenicity of human cervical tumors in nude mice but inhibited the growth of hematopoietic tumors, mastocytoma P815, and plasmocytoma in immunocompetent mice [86, 87].

It is clear that the Th17 cells have an ambiguous role in cancers: they can both encourage and inhibit cancer progression.

It is well established that IL-17 acts as an angiogenic factor that stimulates the migration and cord formation of vascular endothelial cells *in vitro* and elicits vessel formation *in vivo* [88, 89].

The mechanism of Th17 cells upregulation in tumor is not clear. Charles et al. found that TNF-α enhanced tumor growth via the inflammatory cytokine IL-17 in a mouse model of ovarian cancer and in patients with advanced cancer [90]. Su et al. demonstrated that tumor cells and tumor-derived fibroblasts secrete monocyte chemotactic protein 1(MCP-1) and RANTES that mediate the recruitment of Th17 cells [78].

More recently, Kuang et al. showed that tumor-activated monocytes promote expansion of Th17 cells by secreting a set of key proinflammatory cytokines in the peritumoral stroma of hepatocarcinoma tissues [91]. It is clear that Treg cells efficiently suppressed the function of antitumor $CD8^+$ T cells [92, 93]. A recent study reported that IL-2 regulates the balance between tumor Treg and Th17 cells by stimulating the differentiation of the former and inhibiting that of the latter in the tumor microenvironment [76].

The mechanism of Th17 cells' antitumor activity remains largely unknown. One recent work has reported antitumor activity of IL-17 by means of a T-cell-dependent mechanism [87].

Two studies by Benatar et al. demonstrated that IL-17E, a cytokine with significant homology to IL-17, has antitumor activity in multiple tumor models, and that eosinophils and B cells are involved in the antitumor mechanism of action of IL-17E [94, 95].

Th17 cells may contribute to protective human tumor immunity by inducing Th1-type chemokines and stimulating CXCL9 and CXCL10 production to recruit effector cells to the tumor microenvironment. A recent study has also demonstrated that almost half of IL-17-producing $CD4^+$ T cells isolated from hepatocarcinoma tissues simultaneously produced IFN-γ [91].

An interesting work of the Gaudernack group has demonstrated that IL-17-secreting T cell clones obtained from long-term survivors after immunotherapy also secreted IFN-γ, IL-4, IL-5, and IL-13 [96]. More recently, it was shown that Th17 cells and IL-17 participate in antitumor immunity by facilitating dendritic cell recruitment into tumor tissues and promoting the activation of tumor-specific $CD8^+$ T cells [97].

It was even most intriguing that the Th17 frequencies increased during treatment with trastuzumab in patients with breast cancer [82] or with metastatic melanoma treated with the anticytotoxic T lymphocyte-associated antigen 4 (CTLA4) antibody tremelimumab [10].

Alvarez et al. demonstrated that dendritic and tumor cell fusions transduced with adenovirus encoding CD40L eradicate B-cell lymphoma and induce a Th17 type response in a murine lymphoma model [98]. Moreover, Derhovanessian et al. has observed a highly significant correlation between a higher frequency of IL-17-producing T-cells prevaccination and a shorter time to metastatic progression after immunotherapy [99]. These data imply the important involvement of Th17 cells in the response to cancer immunotherapy (Table 1).

Zhang et al. [100] preliminarily reported that compared with healthy volunteers, patients with GC had a higher proportion of Th17 cells in peripheral blood. The increased prevalence of Th17 cells was associated with clinical stage and in advanced disease increased populations of Th17 cells were present also in tumor-draining lymph nodes. Furthermore, the mRNA expression levels of Th17-related factors (IL-17 and IL-23p19) in tumor tissues and the serum concentrations of IL-17 and IL-23 cytokines were significantly increased in patients with advanced GC. The results indicate that Th17 cells may contribute to GC pathogenesis.

5. Concluding Remarks

This paper has highlighted the key roles that T-cell populations play in promotion and/or protection of gastric cancer.

In summary, high densities of cytotoxic T cells and memory T cells are usually associated with favorable survival, indicating the importance of adaptive immunity in the prevention of gastric cancer [41]; as a matter of fact the adoptive immunotherapy of GC with T cells has been also reported in different preclinical and clinical studies [41]. MHC-I restricted CTLs from GC patients recognize tumor-associated antigen and react specifically against self-tumor cells [42, 43], such as MG7-antigen, which shows great potential for predicting early cancer as well as for inducing immune responses to GC [44, 45].

Different studies sometimes reported controversial results, for example, some study showed that Tregs are protective, while others that the Tregs, present in TILs or in peripheral blood of GC patients, are able to suppress the effector T cells, thus promoting the tumor progression [30, 68].

Table 1: Th17 cells in cancer.

Type of cancer	Organism	Role	Effect of subject	Reference
Pancreatic cancer	Mouse	Antitumor	Slower tumor growth and increased survival	Gnerlich et al. [101]
Melanoma	Mouse	Antitumor	Increase in activated $CD8^+$ T cells and better antitumor efficacy	Sharma et al. [102]
Melanoma	Mouse	Antitumor	Th17-polarized cells were better at tumor eradication than Th1-polarized cells	Muranski et al. [103]
Ovarian cancer	Mouse	Pro-tumor	Lead to myeloid cell recruitment in the tumor environment and accelerated tumor growth	Charles et al. [90]
Hepatocellular carcinoma	Mouse	Pro-tumor	Decrease of intratumoral Th17 was associated with decreased tumor growth	Kuang et al. [91]
Prostate cancer	Human	Pro-tumor	Higher pretreatment Th17 numbers correlated with faster disease progression	Derhovanessian et al. [99]
Ovarian cancer	Human	Antitumor	Th17 levels correlated positively with patients survival	Kryczek et al. [82]
Prostate cancer	Human	Antitumor	More highly differentiated Th17 in prostate correlated with slower disease progression	Sfanos et al. [85]
Lung adenocarcinoma	Human	Antitumor	Th17 accumulation correlated positively with patient survival	Ye et al. [104]

In addition, although not conclusively, recent data suggested that Th17 cells might somehow contribute to GC pathogenesis [96].

On the basis of clinical and experimental evidence, it is reasonable to conclude that the T immune response in GC has double faces as Janus, one friend and one foe, and that to obtain successful immunotherapy might involve a combined approach, which intensify the effector functions of cytotoxic T cells and probably reduce the suppressive T cells.

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Clinical Study

Sorafenib Prevents Escape from Host Immunity in Liver Cirrhosis Patients with Advanced Hepatocellular Carcinoma

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Purpose. It has been reported that Th2 cytokines downregulate antitumor immunity, while activation of type T cells promotes antitumor immunity. The aim of this paper was to evaluate host immunity in liver cirrhosis (LC) patients with advanced hepatocellular carcinoma (aHCC) receiving sorafenib therapy. *Methods*. Forty-five adult Japanese LC patients received sorafenib for aHCC between 2009 and 2011 at our hospital. Sorafenib was administered at a dose of 200–800 mg/day for 4 weeks. Blood samples were collected before and after treatment. *Results*. Eleven patients were treated with sorafenib at 200 mg/day (200 group), 27 patients received sorafenib at 400 mg/day (400 group), and 7 patients were given sorafenib at 800 mg/day (800 group). There was no significant change in the percentage of Th1 cells after treatment in any group. However, the percentages of Th2 cells and regulatory T cells were significantly decreased after treatment in the 400 group and 800 group compared with before treatment, although there was no significant change after treatment in the 200 group. *Conclusions*. These results indicate that treatment with sorafenib might induce Th1 dominance and prevent the escape of tumor cells from the host immune system in LC patients with aHCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in men and the eighth most common in women, with over 500,000 new cases being diagnosed worldwide each year [1–3]. Several therapeutic modalities, including surgery, percutaneous ethanol injection (PEI), transcatheter arterial chemoembolization (TACE), and radiofrequency ablation (RFA), are used to treat small tumors. Recently, the oral multikinase inhibitor sorafenib, which shows strong in vitro activity by targeting the Raf/mitogen-activated protein kinase/extracellular signal-related kinase signaling pathway, has been used to treat advanced hepatocellular carcinoma (aHCC). In the Sorafenib HCC Assessment Randomised Protocol (SHARP) study, 602 patients (mainly Europeans) were randomized to receive sorafenib or placebo. They had an Eastern Cooperative Oncology Group performance status of 0–2 and were all in Child-Pugh class A. The sorafenib group achieved a median overall survival time of 10.7 months versus 7.9 months for the placebo group [4]. Sorafenib has also demonstrated significant clinical activity against HCC in phase II and phase III studies [5, 6], in which treatment with this agent achieved a longer median survival time and longer time to radiologic progression compared with placebo.

When treating aHCC in patients with cirrhosis of the liver, we must consider the influence of tumor-related factors, the properties of the anticancer drugs or molecular-targeting agents, and host immunity. Tumors develop various mechanisms to escape from the host immune system and to inhibit antitumor responses. Dendritic cells (DCs) are the most potent antigen-presenting cells with respect to their ability to efficiently prime both CD4-positive and CD8-positive cytotoxic T cells. It has been reported that impaired DC function might be an important factor in allowing tumors to escape from surveillance [7], and that the number of

peripheral blood DCs is significantly decreased in cancer patients [8, 9]. Production of immunosuppressive factors, an increase of regulatory (Treg) cells, and downregulation of the expression of tumor antigens and major histocompatibility complex (MHC) molecules are some of the mechanisms by which tumor cells can escape from immune recognition [10, 11]. All of these mechanisms may operate in patients with HCC. Based on their cytokine production profiles, helper T cells can be divided into two distinct populations, which are known as type 1 helper T cells (Th1 cells) and type 2 helper T cells (Th2 cells). Th1 cells produce interferon-gamma (IFN-gamma) and interleukin 2 (IL-2) and play a pivotal role in cell-mediated immunity, while Th2 cells produce interleukin 4 (IL-4), interleukin 10 (IL-10), and other cytokines that are essential for the regulation of humoral immunity [12, 13]. The Th1 subset is responsible for activation of cell-mediated immunity and cytotoxic $CD8^+$ T lymphocytes (CTLs), while the Th2 subset primarily assists in B cell activation [14]. The direction in which naive $CD4^+$ cells differentiate depends on their first encounter with the triggering agents. The factors regulating differentiation are still not fully understood, although the cytokine environment during the differentiation of antigen-primed $CD4^+$ T helper cells is thought to determine the subset that emerges [15]. IFN-gamma preferentially inhibits the proliferation of Th2 cells, while IL-4 and IL-10 are secreted by Th2 cells and suppress the secretion of IL-12, which is the critical cytokine for Th1 differentiation [16, 17]. Thus, Th1 and Th2 cells cross-regulate their own development. It has been reported that Th2 cytokines inhibit antitumor immunity [18], while the activation of Th1 responses promotes antitumor immunity [19–22]. We have previously shown that Th1 dominance is lost due to an increase of Th2 cells in HCC patients, and that carcinogenesis might be more likely to occur in patients with chronic HCV infection and an increase of Th2 cells [23]. The response of T cells to self-and nonself-antigens is controlled by a network of Treg cells. $CD4^+$ cells that constitutively express CD25, the interleukin-2-receptor α-chain, are generally considered to be natural Treg cells and account for 5–10% of all peripheral $CD4^+$ T cells in healthy animals and humans [24–26].

We previously examined the changes of host immunity and efficacy of treatment in LC patients with aHCC receiving hepatic intra-arterial chemotherapy (HAIC). We found that the percentage of Th2 cells increased in liver cirrhosis (LC) patients with aHCC as the response to HAIC decreased. This suggested that HAIC might be not useful for patients with aHCC because it induces Th2 dominant host immunity [27, 28]. However, it is not clear how sorafenib influences host immunity in LC patients with aHCC. Accordingly, the aim of the present study was to retrospectively evaluate changes of host immunity in LC patients with aHCC receiving sorafenib therapy.

2. Methods

2.1. Patients. Forty-five adult Japanese LC patients were treated for an aHCC with sorafenib between 2009 and 2011 at our hospital. Sorafenib was administered at a dose of 200–800 mg/day for 4 weeks depending on the patient's body habitus and age. Blood samples were collected in the early morning before and after treatment.

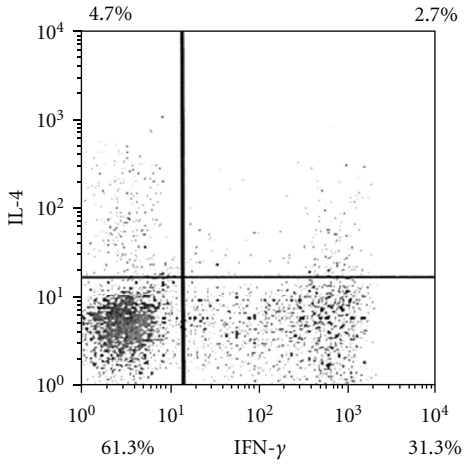
2.2. Analysis of CD4-Positive T Cell Subsets. Peripheral blood CD4-positive T cell subsets were analyzed after nonspecific stimulation with phorbol 12-myristate 13-acetate (PMA), ionomycin, or brefeldin A (Sigma Chemical Co., St. Louis, MO, USA), according to the modified method of Jung et al. [29]. Flow cytometry was used to detect cytoplasmic expression of IFN-gamma and IL-4 by peripheral blood CD4-positive T cells after culture and staining, as reported previously. Results were expressed as the percentage of cytokine-producing cells in the CD4-positive T cell population, which was divided into IFN-gamma-positive/IL-4-negative (Th1) cells and IFN-gamma-negative/IL-4-positive (Th2) cells (Figure 1). Regulatory T cells (Treg cells) were identified as $CD25^{high}/CD127^{low}$ cells (Figure 2).

2.3. Evaluation of Tumor Response. Tumor responses were assessed according to the modified Response Evaluation Criteria in Solid Tumors (RECIST) [30, 31].

2.4. Statistical Analysis. Statistical analysis was performed by using the Statistical Package for the Social Sciences (SPSS version 11.0; SPSS, Chicago, IL, USA). Results are expressed as the mean ± standard deviation (SD). Wilcoxon's signed rank sum test was used to compare patient characteristics within each group. A probability of less than 0.05 was considered to indicate statistical significance in all analyses.

3. Results

The 45 patients were divided into three groups. Eleven patients were administered sorafenib at a dose of 200 mg/day for 4 weeks (200 group), 27 patients were administered 400 mg/day for 4 weeks (400 group), and 7 patients were administered 800 mg/day for 4 weeks (800 group). There were 7 men and 4 women aged 60 to 82 years (mean ± SD: 72.1 ± 7 years) in the 200 group, 24 men and 3 women aged 56 to 79 years (mean ± SD: 69.4 ± 6 years) in the 400 group, and 7 men aged 61 to 80 years (mean ± SD: 66.1 ± 7 years) in the 800 group. In the 200 group, eight patients had HCV-related LC (C-LC), one patient had HBV-related LC (B-LC), and two patients had non-B non-C LC (non-B non-LC), which did not include LC due to autoimmune diseases such as autoimmune hepatitis or primary biliary cirrhosis. In the 400 group, there were 17 patients with C-LC, 5 patients with B-LC, and 5 patients with non B non-C LC. In the 800 group, 1 patient had C-LC, 2 patients had B-LC, and 4 patients had non B non-C LC. The Child-Pugh class was A for 8 patients in the 200 group, 26 patients in the 400 group, and 5 patients in the 800 group, while it was B for 3, 1, and 2 patients, respectively. Nine patients had stage IVA disease and two patients had stage IVB disease in the 200 group. There was 1 patient with stage III disease, 24 patients with stage IVA disease, and 2 patients with stage IVB disease



IFN-γ/IL−4 in CD4

Quad	Events	Gated (%)
UL	293	4.7
UR	166	2.7
LL	3838	61.3
LR	1961	31.3

Figure 1: Flow cytometric detection of interferon (IFN-γ) and interleukin (IL-4) in CD4-positive T cells. Upper left: IFN-γ-negative and IL-4-positive cells (Th2); lower right: IFN-γ-positive and IL-4-negative cells (Th1).

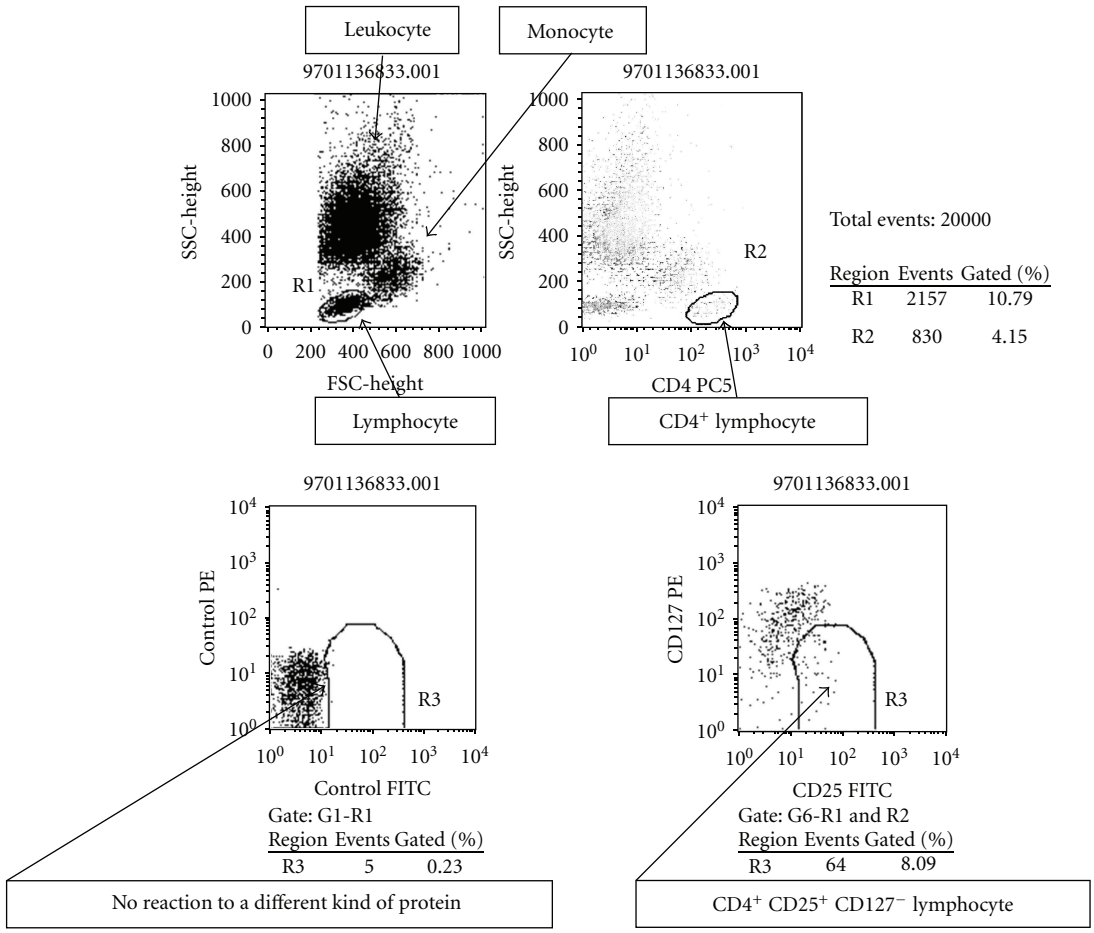


Figure 2: Flow cytometric detection of CD25 FITC and CD127 PE in CD4-positive T cells. Upper left: leucocytes, monocytes, and lymphocytes; Upper right: CD4-positive lymphocytes; lower left: no reaction to a different protein (control); lower right: CD4-positive and CD127-negative lymphocytes (Treg).

Table 1: Clinical characteristics of 45 liver cirrhosis patients with HCC.

Dose of sorafenib	200 mg	400 mg	800 mg
No. of patients	11	27	7
Mean age	72.1 ± 7	69.4 ± 6	66.1 ± 7
Gender (M/F)	7/4	24/3	7/0
Type of cirrhosis (HBV/HCV/non B non C)	1/8/2	5/17/5	2/1/4
Child-Pugh classification (A/B/C)	8/3/0	26/1/0	5/2/0
Stage (III/IVAIIVB)	0/9/2	1/24/2	0/7/0
JIS score (2/3/4/5)	0/8/3/0	0/26/1/0	0/5/2/0

Table 2: Objective responses of liver cirrhosis patients with advanced HCC treated after 4–8 weeks of sorafenib treatment.

Dose of sorafenib	PR	SO	PO	Response rate (%)
200 m g ($n = 11$) (1 dropout)	0	2	8	0.0
400 mg ($n = 27$) (3 dropout)	4	11	9	16.7
800 mg ($n = 7$) (0 dropout)	1	3	3	14.3

in the 400 group, while all 7 patients had stage IVA disease in the 800 group. Eight patients had a Japan Integrated Staging (JIS) score [32] of 3, and three patients had a score of 4 in the 200 group, while the respective numbers were 26 and 1 in the 400 group, as well as 5 and 2 in the 800 group (Table 1). In the 200 group, one patient had involvement of the major branches of the portal vein, and there were no patients with portal trunk thrombus, while the respective numbers were 3 and 4 in the 400 group, as well as 1 and 1 in the 800 group. In the 800 group, one patient had invasion of the main hepatic venous trunk.

3.1. Response. Table 2 summarizes the response to treatment. In the 200 group, 8 of the 11 patients (72.7%) showed progressive disease (PD) and 2 patients (18.2%) had stable disease (SD), but no patient achieved a partial response (PR). In the 400 group, 4 of the 27 patients (14.8%) achieved PR, while 9 patients (33.3%) showed PD and 11 patients (40.7%) had SD. In the 800 group, 1 of the 7 patients (14.3%) achieved PR, while 3 patients (42.9%) patients showed PD and 3 patients (42.9%) had SD.

3.2. Peripheral Blood Th1 and Th2 Cells. There were no significant differences of Th1 cells between before treatment (200 group: 26.3 ± 8%; 400 group: 27.6 ± 11%; 800 group: 27.7 ± 17%) and after treatment (200 group: 23.8 ± 10%; 400 group: 24.9 ± 11%; 800 group: 28.7 ± 18%) in each of the 3 groups (Figure 3). In contrast, significant differences of Th2 cells were noted in the 400 and 800 groups between before treatment (400 group: 3.9 ± 2%; 800 group: 3.7 ± 1%) and

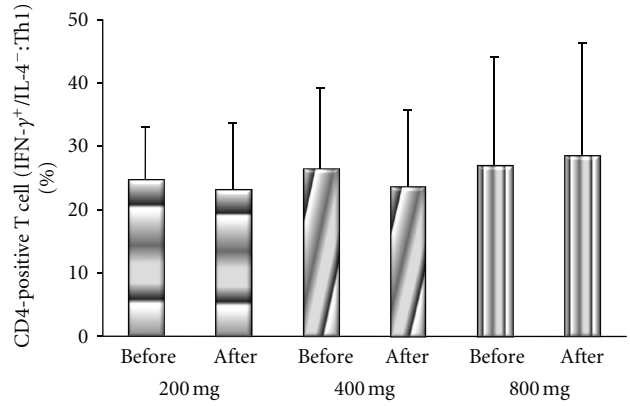


Figure 3: Comparison of the IFN-γ-positive and IL-4-negative (Th1) subset of CD4-positive T cells before and after treatment in the 200 group, 400 group, and 800 group. There were no significant differences between before and after treatment in any group.

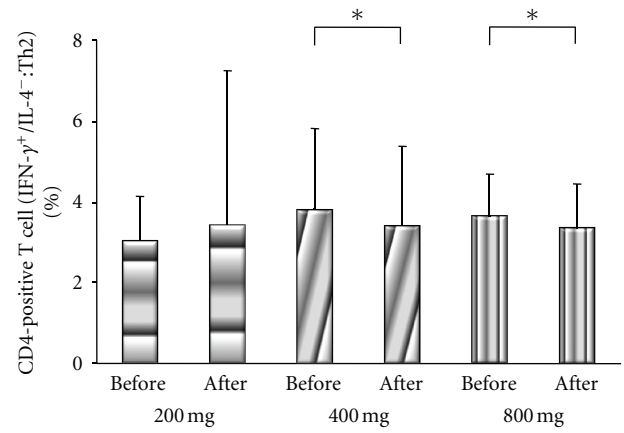


Figure 4: Comparison of the IFN-γ negative and IL-4 positive (Th2) subset of CD4-positive T cells before and after treatment in the 200 group, 400 group, and 800 group. There were significant differences of Th2 cells between before and after treatment in the 400 group and 800 groups ($P < 0.05$ by Wilcoxon's signed rank sum test), but there was no significant difference of 14 Th2 cells in the 200 group.

after treatment (400 group: 3.5 ± 2%; 800 group: 3.3 ± 1%) ($P = 0.014$ and $P = 0.028$, respectively, by Wilcoxon's signed rank sum test), although there was also no significant difference of Th2 cells between before and after treatment (3.1 ± 1% versus 3.3 ± 3%) in the 200 group (Figure 4).

3.3. Peripheral Blood Treg Cells. There were significant differences of Treg cells in the 400 and 800 groups between before treatment (400 group: 9.5 ± 3%; 800 group: 8.5 ± 3%) and after treatment (400 group: 9.2 ± 3%; 800 group: 7.3 ± 3%) ($P = 0.026$ and $P = 0.028$, respectively, by Wilcoxon's signed rank sum test), but there was also no significant difference of Th2 cells between before and after treatment (10.0 ± 2% versus 10.1 ± 3%) in the 200 group (Figure 5).

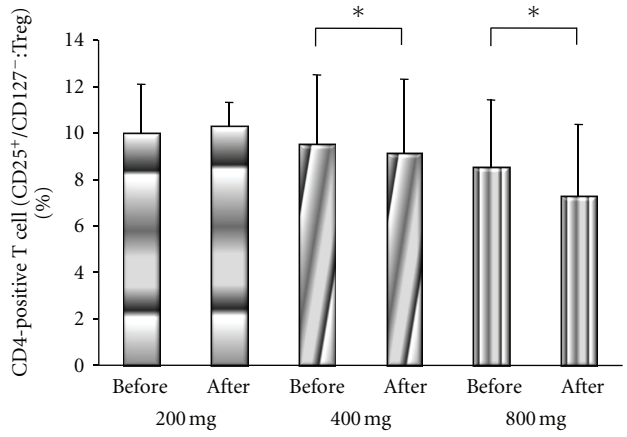


Figure 5: Comparison of CD25 FITC and CD127 PE among CD4-positive T cells (Treg cells) before and after treatment. There were significant differences of Treg cells between before treatment and after treatment in the 400 group and 800 groups ($P < 0.05$ by Wilcoxon's signed rank sum test), but there was no significant difference of Th2 cells in the 200 group.

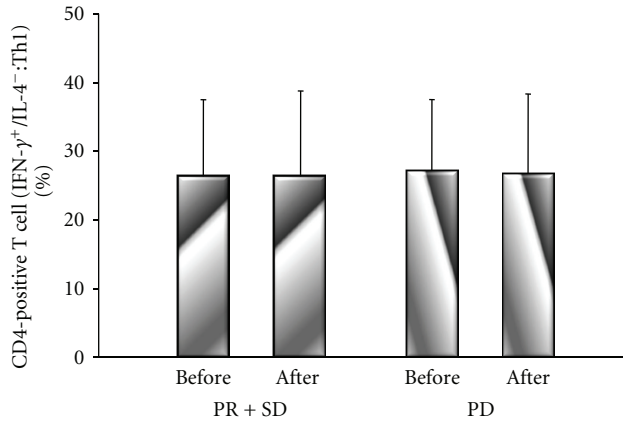


Figure 6: Comparison of the IFN-γ-positive and IL-4-negative (Th1) subset of CD4-positive T cells before and after treatment in the PR + SD group and PD group. There were no significant differences of Th1 cells between before treatment and after treatment in either group.

3.4. Host Immunity and Objective Response. There were no significant differences of Th1 cells between before treatment (PR + SD group: 26.2 ± 11%; PD group: 26.8 + 10%) and after treatment (PR + SD group: 26.2 ± 12%; PD group: 26.3 ± 12%) in either group (Figure 6). However, there were significant differences of Th2 cells in the PR + SD and PD groups between before treatment (PR + SD group: 4.2 ± 2%; PD group: 3.6 ± 1%) and after treatment (PR + SD group: 3.7 ± 2%, $P = 0.017$; PD group: 3.1 ± 1%, $P = 0.020$) (Figure 7). There were no significant differences of Treg cells between before treatment (PR + SD group: 8.9 ± 2%; PD group: 9.6 ± 3%) and after treatment (PR ± SD group: 8.6 ± 2%; PD group: 9.3 ± 3%) in either group, although Treg cells decreased after treatment in both groups 14 (Figure 8).

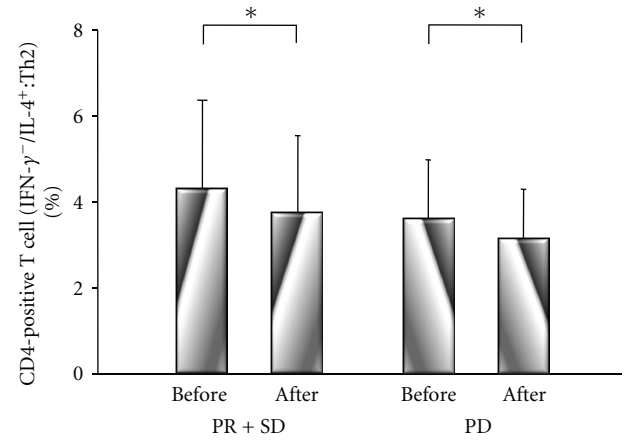


Figure 7: Comparison of the IFN-γ-negative and IL-4-positive (Th2) subset of CD4-positive T cells before and after treatment in the PR + SD group and PD group. There were significant differences of Th2 cells in the PR + SD and PD groups between before treatment and after treatment (PR + SD group: $P = 0.017$, PD group: $P = 0.020$ by Wilcoxon's signed rank sum test).

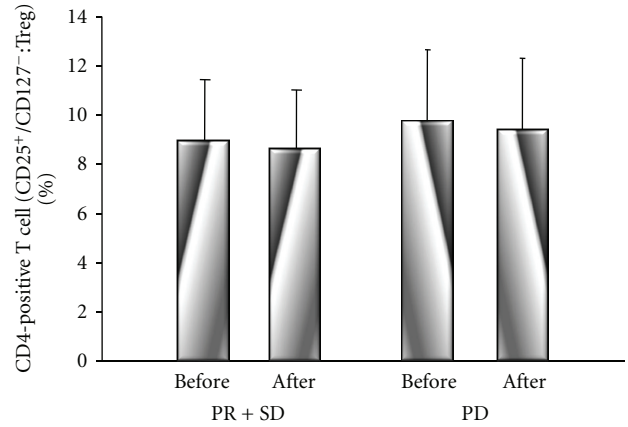


Figure 8: Comparison of CD25 FITC and CD127 PE among CD4-positive T cells (Treg cells) before and after chemotherapy. There were no significant differences of Treg cells between before treatment and after treatment in either group, although Treg cells decreased after treatment in both groups.

4. Discussion

The oral multikinase inhibitor sorafenib has revolutionized the treatment of aHCC in patients with LC. It has been reported that sorafenib therapy prolongs the median overall survival of patients with aHCC [4], but there have been few reports about the influence of sorafenib on host immunity in a HCC patients. Kohga et al. demonstrated that a disintegrin and metalloproteinase 9 (ADAM9) were overexpressed in human HCC tissues, while ADAM9 knockdown increased the expression of membrane-bound MHC class I-related chain A (MICA), decreased the production of soluble MICA, and increased the sensitivity of human HCC cells to natural killer (NK) cells. Furthermore, they indicated that sorafenib enhanced the sensitivity of HCC to NK cells via inhibition of

ADAM9 protease activity and modification of MICA expression [33]. However, it has been unclear whether sorafenib reverses tumor escape mechanisms from host immunity after recognition of MICA expression. Zhao et al. demonstrated that sorafenib inhibited the proliferation of T cells and induced T cell apoptosis and they suggested that sorafenib may impair T cell-related immunity by inducing apoptosis [34]. In addition, Madeleine et al. reported that sorafenib significantly reduced the induction of antigen-specific T cells, impaired the intracellular signaling cascades in DCs, and induced apoptosis of DCs. They concluded that sorafenib interferes with the function and maturation of monocyte-derived DCs [35]. However, it has been unclear whether sorafenib causes similar changes in LC patients with aHCC. The present study showed that there were no significant changes of Th1 cells after treatment in each of the 3 treatment groups. In contrast, the percentage of Th2 cells showed a significant decrease after treatment in the 400 and 800 groups, although there was no significant difference in the 200 group. These results indicate that treatment with sorafenib at doses of 400 mg/day or more can shift host immunity from Th2 dominance to Th1 dominance in LC patients with aHCC, although sorafenib does not increase number of Th1 cells.

There are two distinct subsets of Treg cells in the peripheral lymphoid organs, which are natural Treg (nTreg) cells that develop in the thymus after recognition of high-affinity autoantigens, and induced Treg (iTreg) cells that develop from conventional T cells after peripheral exposure to antigens and cytokines such as TGF-β or IL-10 [36]. These subsets of the Treg network may have a synergistic action or may have different targets that maintain immune homeostasis, although they possibly even have a developmental role [37]. An increase of circulating and tumor-infiltrating FoxP3+ Treg cells has been reported in HCC patients [38]. Sorafenib is the first systemic agent approved for treating HCC and is a multikinase inhibitor with activity against VEGFR2, PDGFR, c-Kit receptor, b-RAF, and p38 [39], which are signal transduction pathways that may be involved in the pathogenesis of HCC [40]. Sorafenib simultaneously inhibits several components of the Raf-MEK-ERK signaling pathway, thus preventing tumor growth and VEGFR-1, VEGFR-2, VEGFR-3, and PDGFR-b, to inhibit neoangiogenesis [41]. In the present study, the percentage of Treg cells in the 400 group and the 800 group showed a significant decrease after treatment compared with before treatment, although there was no significant difference after treatment in the 200 group. These results indicate that sorafenib therapy at doses $\geq$400 mg/day inhibited Treg cells and induced Th1 dominant host immunity in our LC patients with aHCC. It is possible that sorafenib achieved this by decreasing iTreg cells through a reduction of nTreg exposure to HCC antigens by inhibiting tumor neoagiogenesis.

In the present study, the percentage of Th2 cells showed a significant decrease after treatment in both the PR + SD group and the PD group, although there was no significant change of Th1 cells after treatment in either group. In contrast, there were no significant differences of Treg cells between before and after treatment in either group, although these cells decreased after treatment in both groups. These results indicate that treatment-related changes of host immunity in LC patients with aHCC might not influence the objective response to sorafenib.

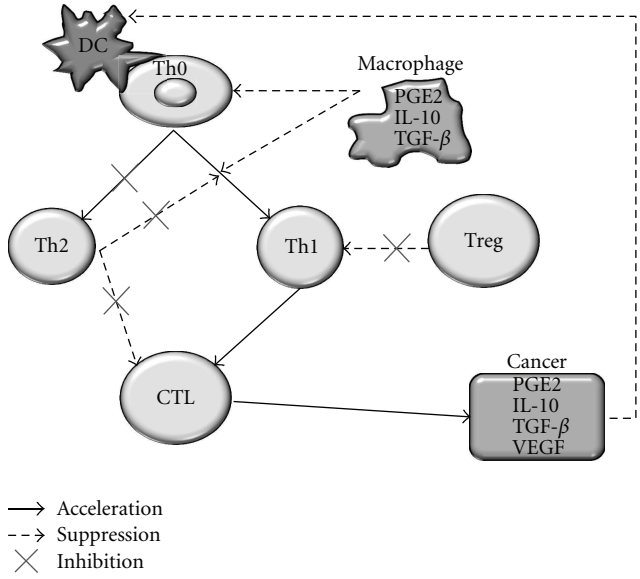


Figure 9: Possible effect of sorafenib on host immunity. Sorafenib therapy might abrogate escape mechanisms from the host immunity in LC patients with aHCC by inducing Th1 dominance. DC: Dendritic cell, Treg: regulatory T cells, Th1: type 1 helper T cells, Th2: type 2 helper T cells, CTL: cytotoxic $CD8^+$ T lymphocytes.

In conclusion, we demonstrated that administration of sorafenib at doses >400 mg/day induced Th1 dominant host immunity in LC patients with aHCC. This effect of sorafenib therapy might be dependent on two mechanisms, which are (1) induction of antigen-primed $CD4^+$ T helper cells after recognition of MICA expression by HCC cells and (2) a decrease of Treg cells related to inhibition of tumor neoangiogenesis. It is also possible that sorafenib might induce T cell apoptosis or interfere with the function and maturation of monocyte-derived DCs. Sorafenib therapy at doses >400 mg/day has the potential to abrogate the mechanisms of tumor escape from the host immune system in LC patients with aHCC by inducing Th1 dominance (Figure 9). Accordingly, neoadjuvant therapy with sorafenib before induction of chemotherapy might prolong the survival or improve the objective response of LC patients with aHCC receiving HAIC by modifying host immunity.

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Research Article

Immunological and Nonimmunological Effects of Indoleamine 2,3-Dioxygenase on Breast Tumor Growth and Spontaneous Metastasis Formation

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The role of the tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO1), in tumor escape and metastasis formation was analyzed using two pairs of *Ido1*+ and *Ido1*− murine breast cancer cell lines. *Ido1* expression in 4T1 cells was knocked down by shRNA, and *Ido1* expression in NT-5 cells was upregulated by stable transfection. Growth of *Ido1*− tumors and spontaneous metastasis formation were inhibited in immunocompetent mice. A higher level of cytotoxic T lymphocytes was generated by spleen cells from mice bearing *Ido1*− tumors than *Ido1*+ tumors. Tumor and metastatic growth was enhanced in immunodeficient mice, confirming an intensified immune response in the absence of *Ido1* expression. However, *Ido1*+ tumors grow faster than *Ido1*− tumors in immunodeficient SCID/beige mice (lacking T, B, and NK cells) suggesting that some *Ido1*-controlled nonimmunological mechanisms may be involved in tumor cell growth regulation. *In vitro* experiments demonstrated that downregulation of *Ido1* in tumor cells was associated with decreased cell proliferation, increased apoptosis, and changed expression of cell cycle regulatory genes, whereas upregulation of *Ido1* in the cells had the opposite effects. Taken together, our findings indicate that *Ido1* expression could exert immunological and nonimmunological effects in murine breast tumor cells.

1. Introduction

Immune escape is one of the hallmarks of cancer [1]. There are a variety of active mechanisms of immune suppression that are elaborated by tumors to drive immune escape, such as the loss of MHC class I molecules or tumor antigens, induction of T regulatory cells, and the production of various immunosuppressive molecules (IL-10, TGF-β, adenosine, PGE2, etc.) [2–4]. One mechanism of immune escape that has been linked to cancer is elevation of the tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO1) [5–14].

IDO1 can be expressed in many tissue and cell types, such as placenta, lung, gut, and epididymis [13, 15–17]. The expression of IDO1 at the maternal-fetal interface in mice placenta is believed to play a role in the protection of the fetus from immunologic rejection by maternal immune mechanisms [18]. Indeed, this has been supported experimentally wherein the pharmacological inhibition of IDO1 by 1-methyltryptophan (1MT) resulted in the rejection of allogeneic, but not syngeneic, murine fetuses [19–21]. *Ido1* activation occurs commonly in tumor cells and/or tumor-draining lymph nodes (TDLNs), and pharmacological inhibition of IDO1 with 1-MT has been shown to result in T-cell-dependent antitumor responses in animal models [8, 22–27]. 1-MT was observed to retard tumor outgrowth but was unable to trigger complete tumor regression [6]. *In vitro* studies indicate that IDO1 is capable of exerting suppressive effects directly on T cells and can activate suppressive populations of regulatory T cells [8, 9]. Furthermore, soluble cytotoxic-T-lymphocyte-antigen-4- (CTLA4-) expressing T regulatory cells induce IDO1 expression in DC, converting them into regulatory antigen-presenting cells (APCs) [24, 26]. Intracellular signaling via CD80/86, CD200R, and FcεRI

could induce IDO1 expression by DC [9, 13, 25]. A tumor-related immune escape mechanism based on tryptophan degradation by IDO1 has been proposed [22, 28]. *Ido1* is expressed by tumor cells; however, the level of *Ido1* expression is significantly lower than in placenta. Tumor cell inhibition of immune response was only demonstrated for *Ido1*-transfected clones, exhibiting 1000-fold increased expression of *Ido1* mRNA relative to placental levels [22, 29]. Thus, a role for IDO1 in tumor immune response is indicated but requires further investigation.

In this study, we examined the impact of IDO1 on tumor growth and metastasis in immune-competent and immune-deficient mice. Two murine breast cell lines, 4T1 and NT-5, expressing *Ido1* and missing *Ido1* expression, respectively, were utilized. NT-5 cells were transfected with an *Ido1* expression vector to establish an NT-5/*Ido1*+ line. Expression of *Ido1* in 4T1 cells was knocked down by transfection with an *Ido1*− specific shRNA expressing plasmid to establish a 4T1/*Ido1*− line. Using these two pairs of cell lines, we examined the relationship between *Ido1* expression and cancer cell growth *in vitro* and *in vivo*. Our analysis of tumor growth and metastasis, in immunocompetent and immunodeficient mice, revealed that IDO1 not only modulated the immunological system, but also played an important biological role in tumor cell proliferation, cell cycle regulation, and anti-apoptotic signaling.

2. Materials and Methods

2.1. Tumor Cell Lines. The NT-5 HER-2/neu-expressing tumor cell line was provided by Elizabeth Jaffe, John Hopkins University. The 4T1 mouse mammary tumor cell line was purchased from American Type Culture Collection. Cells were cultured in RPMI-1640 medium (Cellgro Mediatech, Inc, Manassas, VA) supplemented with 10% FBS (Sigma-Aldrich Co, St. Luis, MO).

2.2. Plasmid Construction and Cell Transfection. The mammalian expression vector for *Ido1* was constructed by inserting full-size mouse *Ido1* cDNA into the vector pRc/CMV (Invitrogen, Life Technologies Corp., Carlsbad, CA). NT-5 cells were cloned, and IDO expression in the individual clones was evaluated. The clone with the lowest IDO1 expression was used for transfection with either *Ido1* constructs or control pRc/CMV vector using Lipofectamine 2000 according to manufacturer instructions (Invitrogen). Stable transfectants (NT-5/*Ido1*+ and NT-5/vector) were selected by growth in a medium supplemented with 400 μg/mL G418 (Sigma-Aldrich Co). IDO-positive 4T1 breast tumor cells were also cloned, and the clone with highest IDO expression was transfected with shRNA against IDO containing plasmid and the control plasmid (SABiosences). Stably transfected 4T1/*Ido1*− and 4T1/vector cells were selected with 600 μg/mL of G418 (Sigma-Aldrich).

2.3. Real-Time PCR and Gene Expression Profiling. RNA was isolated from the cells using the RNeasy kit from Qiagen (Valencia, CA), and the extracted RNA was converted to cDNA using the RT^2 First Strand Kit from Super Array Bioscience Corporation (Frederick, MD) according to manufacturer's protocols. Real-time PCR was carried out using the Super Array RT^2 Real-Time SYBR Green PCR Master Mix (Super Array Bioscience) and was performed on the ABI Prism 7700 sequence detector real-time PCR system (AB Applied Biosystems, Foster City, CA). Primers for mouse *Ido1* were forward 5′-GTACATCACCATGGCGTATG-3′; reverse: 5′-CGAGGAAGAAGCCCTTGTC-3′. Standard curves were generated from five 10-fold serial dilutions of tumor cell cDNA, and no product could be observed in the negative control lacking template. Differences in gene expression were calculated by using the $\triangle$Ct method and normalized to GAPDH according to the manual from Super Array Bioscience (Super Array, Bioscience Corp., Frederick, MD).

The RT^2 Profiler PCR Array System and mouse cell cycle regulation RT^2 Profiler PCR Array (Super Array, Bioscience Corp) were used. Real-time PCR detection was carried out per the manufacturer's instructions. The experimental cocktail was prepared by mixing cDNA isolated from cell lines with the RT^2 Real-Time SyBR Green/ROX qPCR Master Mix (SABiosciences Corp., Frederick, MD). The mixtures were equally aliquoted into the 96-well plate containing predispensed gene-specific primer sets, then real-time PCR was performed using the ABI Prism 7900HT (ABI, Applied Biosytems Corp., Foster City, CA). Differences in genes expression were compared between 4T1/vector and 4T1/*Ido1*− cells or between NT-5/vector and NT-5/*Ido1*+ cells. Analyses of the raw data were done through the Super Array Data Analysis Web Portal (Super Array Bioscience Corp.).

2.4. Enzymatic Assay for IDO Activity. IDO activity was determined using what is described in [10, 30]. Briefly, cell extracts were prepared, mixed with an equal volume of 2x reaction buffer (100 mM PBS, 40 mM ascorbate, 20 μM methylene blue, 200 μg/mL catalase, 800 μM L-Tryptophan (Affymetrix, USB Inc, Cleveland, OH), pH 6.5), and incubated at 37°C for 30 min in order to permit IDO-mediated conversion of L-Trp into N-formylkynurenine. The reaction was stopped with 30% trichloroacetic acid, and samples were incubated at 50°C for 30 min to hydrolyze the N-formylkynurenine produced to kynurenine. After centrifugation, supernatants were collected and mixed with Ehrlich's reagent (0.8% p-dimethylaminobenzaldehyde in acetic acid) in 96-well plates, and absorbance at 490 nm was measured on a microplate reader. IDO activity is defined as the amount of enzyme required to produce 1 nmol of kynurenine per hr per mg of protein.

2.5. In Vitro Cell Proliferation Assay. The cells were cultured in a 96-well plate for 2 days and pulsed with 0.2 μCi/well of ^{3}H-thymidine overnight followed by collection of supernatant. 50 uL of supernatant was mixed with 2 mL of Cyto-Spin (MP, Biomedicals LLC, Solon, OH) and counted by liquid scintillation counter (Wallac). The tumor cells were also counted by Cellomics Array Scan HCS Reader (Thermo Fisher, Pittsburgh, PA) following 3 days in culture as described previously [31].

2.6. Flow Cytometry Analysis. Cells were cultured in a control medium or in serum starvation conditions (RPMI with 0.1% FBS or 0.3% FBS) for 3 days followed by culture in RPMI with 10.0% FBS for 24 hours. Cells were stained with FITC-conjugated antibromodeoxyuridine (BrdU) and/or propidium iodide (PI) using FITC BrdU Flow Kit (BD Biosciences, Pharmingen, San Diego, CA) according to the manufacturer's instruction. Flow cytometry was performed by CYAN ADP (Beckman Counter, Brea, CA). The data were analyzed with the Cytomation Summit v4.3 software.

2.7. In Vivo Local and Metastatic Tumors Assays. BALB/c, FVB/N, and SCID-beige were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 8-9 weeks of age. Mice were housed in the University of Pittsburgh Cancer Institute animal facility which is accredited for animal care by the American Association of Laboratory Animal Care. Experiments were performed in accordance with the approved institutional protocol and the guidelines of the Institutional Animal Care and Use Committee. BALB/c and SCID-beige mice were inoculated s.c. with 2.5×10^4 or 1.0×10^5 of 4T1/vector cells or 4T1/*Ido1*− cells per mouse; FBV/N and SCID-beige mice were inoculated s.c. with 5×10^6 of NT-5 or NT-5/*Ido1*+ cells per mouse. Tumor growth was evaluated by measurement of tumor diameters 3 times a week, and the tumor volume was calculated as length $\times$ width$^2 \times$ 0.52. All data is represented as mean ± SE. Experiments were terminated when tumors reached 2.0 cm in diameter. Each group contained 10 mice. Experiments were repeated twice. Lungs were harvested when tumors reached approximately 2 cm in diameter and fixed in Bounce solution. Metastases were counted under a dissecting microscope.

2.8. Cytotoxicity Assay. Spleen cells from BALB/c mice bearing 4T1/*Ido1*− or 4T1 tumors were cultured with irradiated (15,000 rad) 4T1/*Ido1*− or 4T1 tumor cells for 5–8 days in the presence of 300 IU/mL of IL-2 in 24-well plates at the ratio as 3×10^6 spleen cells: 1×10^6 tumor cells. The cytotoxic activity of restimulated spleen cells was tested against ^{51}Cr-labeled 4T1/*Ido1*− or 4T1 cells at the effector: target ratio as 100 : 1. Spleen cells were distributed into V-96-well plates preloaded with ^{51}Cr-labeled tumor cells; 96-well plates containing cell mixtures were centrifuged at 2000 rpm for 2 minutes and then incubated 4 hours at 37°C. 25 μL of supernatant was transferred into yttrium silicate scintillator-coated microplates (LumaPlate-96, PerkinElmer) and left overnight at RT. The level of β-emission released by ^{51}Cr was measured in a β-counter.

2.9. Western Blot Analysis. The cells were lysed in RIPA buffer supplemented with 1% CLAP cocktail (antipan, leupeptin, pepstatin, and chymostatin) and 1 mM PMSF. 50 ug of protein extracts were resolved on 4.5–12% of SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% nonfat dry milk in TBST buffer, probed with antibodies against stratifin (1 : 200, Sigma), and Atm (1 : 200, Santa Cruz Biotechnology, Inc, Santa Cruz, CA) for two hours at RT or overnight at 4°C, and then incubated with horseradish peroxidase-labeled secondary antibody (Santa Cruz). The signals were detected by ECL (Amersham). Films were scanned and analyzed by Image-QuanT data analysis software (Molecular Dynamics).

2.10. Statistical Analysis. Data are presented as mean ± SD. Comparisons between values were performed using a two-tailed Student's *t*-test. For the comparison of multiple groups, a one- or two-way ANOVA test was applied. For all statistical analyses, the level of significance was set at a pro bability of $P < 0.05$. All experiments were repeated 2-3 times.

3. Results

3.1. Establishment of Two-Paired Stable Clones (Ido1− and Ido1+) of Murine Breast Cancer Cells. Initially, we measured *Ido1* expression in a panel of mouse tumor cell lines of differing histological origin. Two mouse breast tumor cell lines, 4T1 and NT-5, which showed differential expression of *Ido1*, were chosen. 4T1 cells, derived from sporadic breast tumor in BALB/c mice, are highly aggressive, metastatic, and poorly immunogenic [32, 33]. NT-5 breast tumor cells are derived from HER-2/*neu*-transgenic FBV/N mice, and they are immunogenic [34].

We found that 4T1 cells express *Ido1*, whereas the level of *Ido1* gene expression in NT-5 cells was very low (*Ido1*−). Clonal variation could be attributable whenever transfection is performed on a polyclonal whole cell population. To avoid this, our transfections were designed as follows: 4T1 breast tumor cells were cloned using a standard approach for selecting single-cell clones. *Ido1* expression, in these individual cell clones, was evaluated. The clone the of the 4T1 tumor cells with the highest *Ido1* expression was transfected with *Ido1* siRNA and a control vector to generate stable 4T1/*Ido1*− and 4T1/vector clones.

Similarly, NT-5 cells (expressing *Ido1* at a very low level) were also cloned, and the clone of NT-5 cells, with the lowest level of *Ido1* expression, was selected for transfection with cDNA encoding *Ido1* or control plasmid.

As shown in Figure 1(a), very low *Ido1* expression was detected by RT-PCR in 4T1/*Ido1*− cells compared with 4T1 and 4T1/vector cells. The quantitative RT-PCR analysis revealed 78% downregulation of *Ido1* in 4T1/*Ido1*− cells, and no changes of *Ido1* expression in 4T1/vector cells in comparison with naïve 4T1 cells (Figure 1(a)).

IFN-γ is a known inducer of *Ido1* [30]. We tested whether *Ido1* expression could be induced by IFN-γ in 4T1, 4T1/vector, and 4T1/*Ido1*− cells. *Ido1* expression was strongly increased (30-fold change) in 4T1, and 4T1/vector cells were treated with IFN-γ (Figure 1(b)). However, *Ido1* activation was abrogated in shRNA-transfected cells (4T1/*Ido1*−) (Figure 1(b)).

We transfected an *Ido1* vector and scramble vector into the *Ido1* low-expressing NT-5 cells and established stable *Ido1* expressing NT-5/*Ido1*+ and NT-5/vector clones. RT-PCR analysis showed *Ido1* expression in NT-5/*Ido1*+ cells, and very low expression in NT-5/vector cells (Figure 1(c) top); quantitative RT-PCR demonstrated a 50.9-fold increase

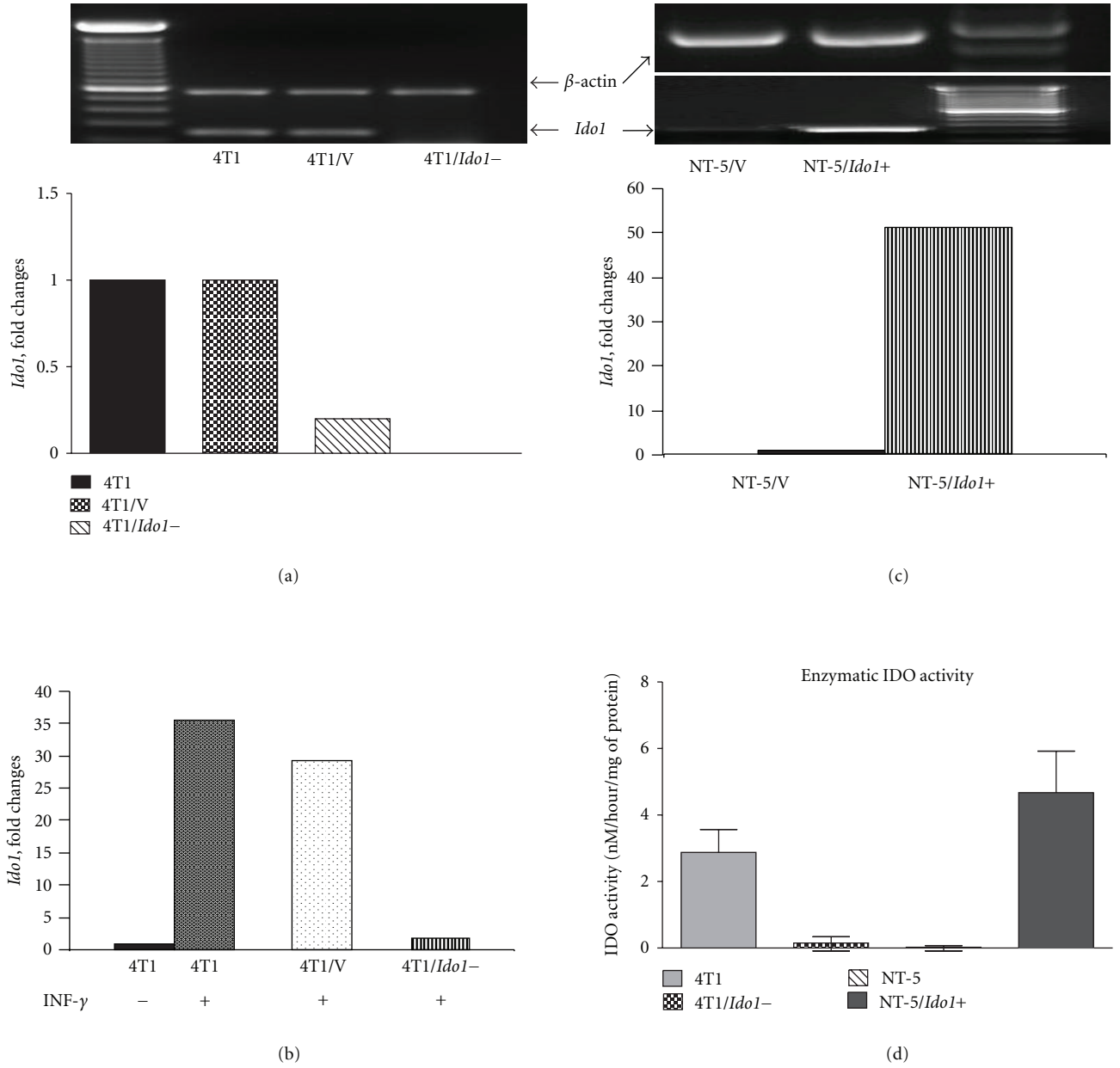

Figure 1: Generation of two pairs of *Ido1*+ and *Ido1*− clones from murine breast cancer cells. (a) RT-PCR (top picture) real-time PCR (bottom picture) analysis of *Ido1* expression in 4T1 cells, cells transfected with vector (4T1/V), and cells transfected with shRNA for *Ido1* (4T1/*Ido1*−). Fold change was compared with 4T1. (b) Real-time PCR analysis of the effect of INF-γ treatment (INF-γ, 25 ng/mL, 24 hours) on *Ido1* expression in 4T1, 4T1/V, and 4T1/*Ido1*− cells. (c) *Ido1* expression in NT-5 cells transfected with vector (NT-5/V) and cells transfected with *Ido1* cDNA (NT-5/*Ido1*+) detected by RT-PCR (top picture) and by Real-time PCR (bottom picture). (d) Enzymatic IDO activity in 4T1, 4T1/*Ido1*−, NT-5, and NT-5/*Ido1*+ cells. IDO activity is defined as the amount of enzyme required to produce 1 nmol of kynurenine per hr per 1 mg of protein. All values are the means of four measurements.

of *Ido1* expression in NT-5/*Ido1*+ cells in comparison to NT-5/vector cells (Figure 1(c)).

Next, we tested enzymatic activity of IDO in 4T1, 4T1/*Ido1*− NT-5, and NT-5/*Ido1*+ cells (Figure 1(d)). 4T1/*Ido1*− cells demonstrated a significant reduction of IDO activity in comparison with 4T1 cells, while NT-5/*Ido1*+ cells showed a significant increase in IDO activity when compared with NT-5 cells (Figure 1(d)).

3.2. Effect of Ido1 Expression on Tumor Growth and Metastasis Formation. To determine the role of IDO1 in tumor growth, BALB/c mice were inoculated s.c. with 4T1/vector or 4T1/*Ido1*− tumor cells. 4T1/*Ido1*− tumors grew significantly ($P < 0.05$) slower than 4T1/vector tumors (Figure 2(a)). Mice bearing 2 cm tumors were sacrificed according to our IACUC-approved protocol, and surviving mice bearing tumors of less than 2 cm were counted at sequential time

Table 1: Spontaneous lung metastases formation by 4T1/*Ido1*+, 4T1/*Ido1*− or NT-5/*Ido1*+, NT-5/*Ido1*− tumors in immunocompetent and immunodeficient mice.

Mice[1]	No. of spontaneous pulmonary metastases	
	4T1/*Ido1*+ tumors	4T1/*Ido1*− tumors
BALB/c	56; (41, 51, 56, 66, 182)	21; (6, 12, 21, 24, 29)*
SCID-beige	54; (23, 24, 54, 75, 164)	69; (33, 54, 64, 69, 69, 71, 77, 92)
Mice[1]	NT-5/*Ido1*+ tumors	NT-5/*Ido1*− tumors
FVB/N	6; (0, 2, 3, 5, 6, 7, 8, 11, 14)	1.5; (0, 0, 0, 0, 3, 4, 8, 10)*
SCID-beige	25; (11, 12, 16, 24, 25, 26, 28, 32, 44)*	13; (0, 3, 12, 13, 13, 13, 14, 15)

[1] BALB/c and SCID-beige were inoculated s.c. with 2.5×10^4 4T1/*Ido1*+ or 4T1/*Ido1*− tumor cells. FVB/N and SCID-beige were inoculated s.c. with 5×10^6 NT-5/*Ido1*+ or NT-5/*Ido1*− tumor cells. When tumors reached about 2 cm in diameter, lungs were harvested and fixed in the Bounce solution. Metastases were counted under dissecting microscope. *Significantly ($P < 0.05$) differ from all other groups.

points in order to evaluate survival. At the time of complete mortality in the control group, we observed nearly 50% survival of mice bearing *Ido1*− tumors (Figure 2(b)).

The elevated survival of mice bearing 4T1/*Ido1*− tumors may be a result of a less efficient immune response against tumor cells demonstrating inhibited *Ido1* expression. To assess the immune response of BALB/c mice to 4T1/vector and 4T1/*Ido1*− tumors, spleens of mice bearing tumors were harvested, and spleen cell suspensions were prepared. Freshly prepared spleen cells had no cytotoxic activity against 4T1 tumor cells. Therefore, spleen cells were stimulated with irradiated 4T1 tumor cells for 5–8 days in the presence of IL-2 (300 IU/mL). We observed no difference in the abilities of irradiated 4T1/*Ido1*− and 4T1 (*Ido1*+) cells to stimulate spleen cells and generate cytotoxic T cells. The cytotoxic activity of stimulated spleen cells appeared after 5 days of stimulation and became more prominent after 7-8 days. Spleen cells obtained from irradiated 4T1/*Ido1* tumor cells from 4T1/*Ido1*− tumor bearing mice demonstrated a higher cytotoxic activity than spleen cells from mice bearing 4T1/vector tumors (Figure 2(c)). Furthermore, targeted 4T1/*Ido1*− tumor cells were more sensitive to the cytotoxic activity of spleen cells than 4T1/vector cells (Figure 2(c)). Spleen cells cultured in IL-2 (300 IU/mL) without irradiated tumor cells showed no cytotoxic activity (data no shown). These results indicate that inhibition of 4T1/*Ido1*− tumor growth in BALB/c mice could be due to an elevated immune response against these cells as well as a greater sensitivity of 4T1/*Ido1*− cells to immune destruction.

To further test whether 4T1/*Ido1*− tumor growth inhibition is immunologically mediated, immunocompetent BALB/c mice or immunodeficient SCID-beige mice (which lack T, B, and NK cells) were inoculated s.c. with 4T1/vector and 4T1/*Ido1*− cells. Our expectation was that if differences in *Ido1*+ and *Ido1*− tumor growth are immune modulated, then both tumors should grow similarly in SCID/beige mice. Tumor growth from *Ido1*− cells was inhibited in comparison with *Ido1*+ cells in BALB/c mice. As shown in Figure 2(d), both *Ido1*− and *Ido1*+ tumors grew faster in immunodeficient SCID/beige mice than in immunocompetent BALB/c mice. However, *Ido1*+ tumors grew faster than *Ido1*− tumors in SCID/beige mice suggesting that some *Ido1*-controlled nonimmunological mechanisms may be involved in tumor cell growth regulation.

To test whether spontaneous pulmonary metastasis formation is dependent on *Ido1* gene expression, the number of metastases in BALB/c and SCID/beige mice was counted when tumors reached 2 cm in diameter. We found that 4T1/*Ido1*− cells generated significantly less metastasis in the lungs of BALB/c mice than 4T1 cells (92 versus 396) (Table 1). The pulmonary metastases observed in immunodeficient SCID-beige mice bearing *Ido1*− tumors were higher than in the immunocompetent BALB/c mice bearing *Ido1*− tumors (Table 1).

To further elucidate the role of IDO1, in tumor growth and metastasis formation, we used NT-5/*Ido1*− cells and NT-5/*Ido1*+ tumor cells and FVB/N mice that are immunocompetent for NT-5 cells. It is known that NT-5 breast tumor cells, derived from HER-2/*neu*-transgenic FBV/N mice, are immunogenic [34]. Indeed, tumor growth was prevented when 1×10^6 NT-5 cells were inoculated into mice. However, inoculation of 5×10^6 NT-5 cells resulted in tumor development. Tumor growth in many FVB/N mice was associated with enormous necrosis which made it difficult to measure tumor size; therefore, mice were sacrificed when they showed signs of moribund, and lung metastases were counted. We found that NT-5/*Ido1*+ cells are more metastatic than NT-5/*Ido*−cells in immunocompetent FVB/N mice (Table 1). The numbers of metastases developed from NT-5/*Ido1*+ cells and NT-5/*Ido*− cells in immunodeficient SCID/beige mice were significantly higher than in FVB/mice (Table 1). However, even in immunodeficient mice, NT-5/*Ido1*− cells developed less metastasis than NT-5/*Ido1*+ cells.

Taken together, these results indicate that *Ido1* plays role in tumor growth and metastasis formation.

3.3. Effect of Ido1 Expression on Apoptosis Changes Cell Cycle and Tumor Cell Proliferation In Vitro. The differences in *Ido1*+ and *Ido1*− tumor growth in immunodeficient mice indicate a possible role of *Ido1* in apoptosis and tumor cell proliferation. To analyze cell cycle distribution, in *Ido1*+ and *Ido1*− cells, we used flow cytometry and FITC-conjugated antibromodeoxyuridine (BrdU) and/or propidium iodide (PI) staining. The approach relies on the detection of 5′-bromo-2′-deoxyuridine (BrdU) incorporation which identifies DNA-replicating cells, and PI staining reveals the distribution of cells in three major phases of the cycle (G1 ver-

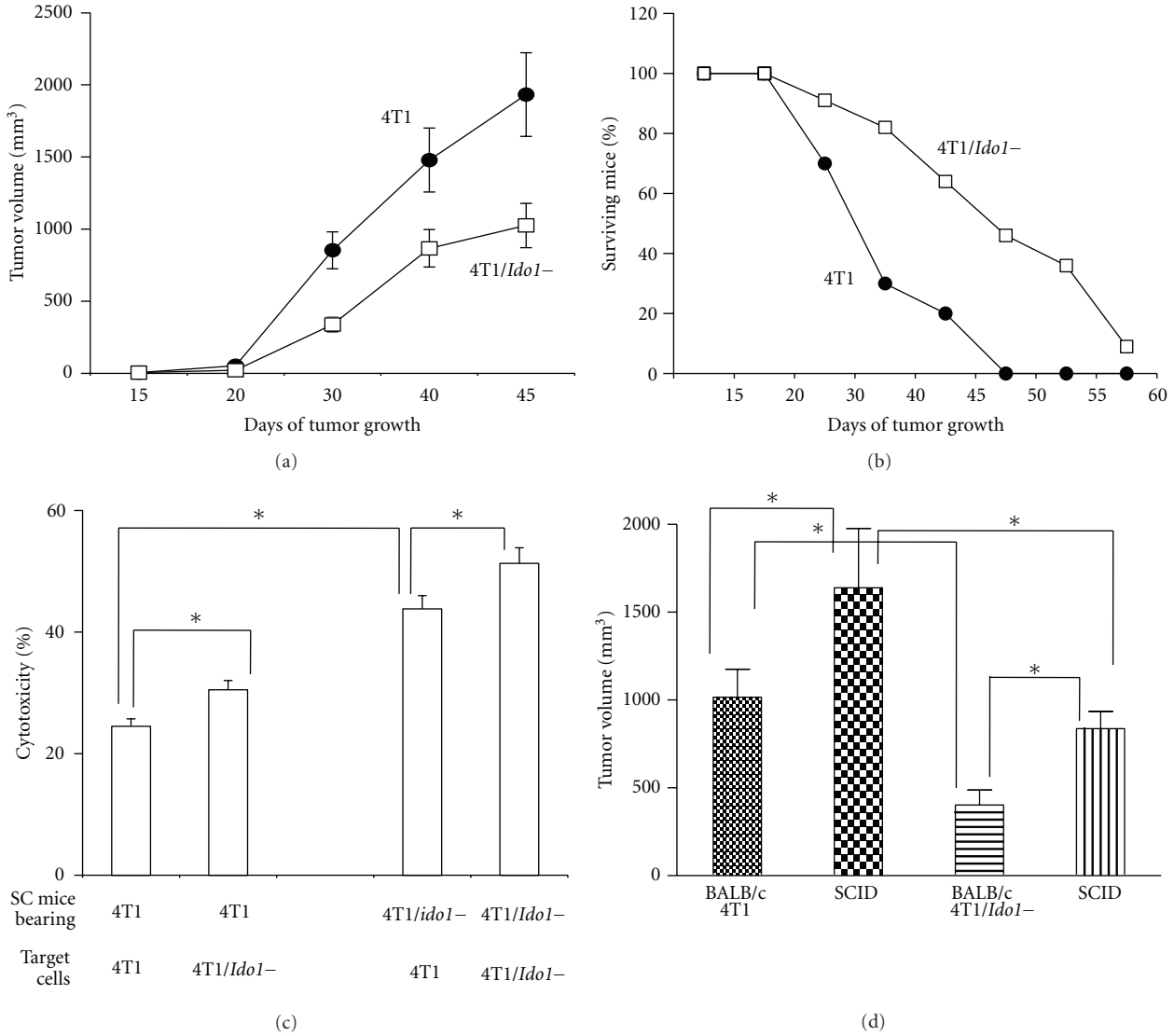

Figure 2: Effect of *Ido1* expression on tumor growth and spontaneous metastasis formation. (a) BALB/c mice were inoculated s.c. with 1×10^5cells/mouse of 4T1/vector or 4T1/*Ido1*− tumor cells, and tumor growth was monitored. (b) Percentage of surviving mice bearing 4T1/vector and 4T1/*Ido1*− tumors. (c) Cytotoxic activity of spleen cells from mice bearing 4T1 or 4T1/*Ido1*− tumors against 4T1 and 4T1/*Ido1*− tumor cells. Spleen cells from mice bearing 4T1/vector or 4T1/*Ido1*− tumors were cultured with irradiated 4T1/vector tumor cells and 300 IU/mL of IL-2 for 5–8 days. The cytotoxic activity of the spleen cells was tested against ^{51}Cr-labeled 4T1 or 4T1/*Ido1*− cells at the E : T ratio 100 : 1. (d) Growth of 4T1 and 4T1/*Ido1*− tumors in immunocompetent BALB/c and immunodeficient SCID-beige mice.

sus S versus G2/M) and makes it possible to detect apoptotic cells with fractional DNA content.

Figure 3 represents the results of the experiments in which double staining was applied. Bivariate distribution (contour map) of DNA content and BrdU incorporation in 4T1 and 4T1/*Ido1*− cells are shown. The 4T1/*Ido1*− cells growing in control media have significantly ($P < 0.05$) higher percentage of apoptotic cells than 4T1/*Ido1*+ cells. Starvation is a condition that often occurs within the tumors. We analyzed the effect of starvation conditions (low serum in culture medium) on apoptosis induction in 4T1/*Ido1*+ and 4T1/*Ido1*− cells. Here, the proportion of apoptotic 4T1/*Ido1*− cells reached 86%, which is approximately 3 times higher than that observed in the 4T1/*Ido1*+ cell population. The 4T1/*Ido1*+ cells recover faster after starvation (Figure 3). Cell cycle phase distribution was also different in 4T1/*Ido1*+ and 4T1/*Ido1*− cell populations in control media and under starvation (Figure 3). Control cells showed a significantly higher proportion of cells in the S phase than 4T1/*Ido1*− cells. 4T1/*Ido1*+ growing in low serum demonstrated a higher proportion of cells in M and G0 phases than 4T1/*Ido1*− cells. We then compared the proliferation of 4T1/*Ido1*+ and 4T1/*Ido1*− cells as well as NT-5/*Ido1*− and NT-5/*Ido1*+ cells *in vitro*. The results of ^{3}H-thymidine incorporation showed a significant reduction in proliferation of *Ido1*− cells in comparison to *Ido1*+ cells (Figure 4(a)). Next, the same pairs of *Ido1*+/*Ido1*− cells were grown for 3 days and counted as described previously [31]. As shown in Figure 4(b),

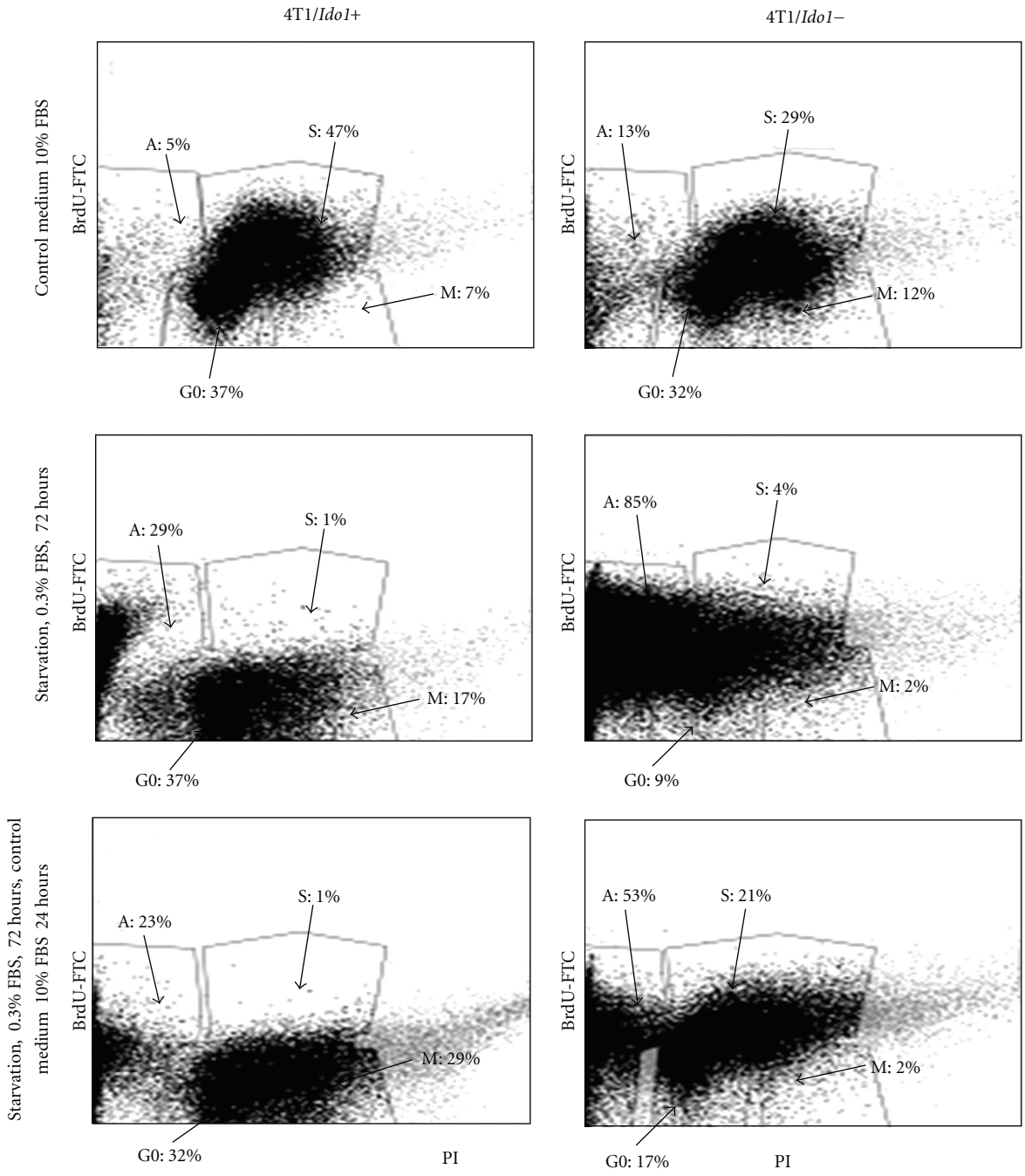

Figure 3: Flow cytometry analysis of apoptosis and cell cycle in *Ido1*+ and *Ido1*− cells. 4T1 and 4T1/*Ido1*− cells were cultured in normal medium (10% FBS), or under starvation condition (0.3% FBS) for 3 days followed by a 24 hour recovery in normal medium. Flow cytometry analyses of cells stained with FITC-conjugated antibromodeoxyuridine (BrdU) and propidium iodide (PI) were performed. The data are presented as a percentage of cells in apoptosis (A), G0, S, and M phases.

the inhibition of *Ido1* activity in 4T1 cells was associated with an inhibition in 4T1/*Ido1*− cell proliferation, whereas overexpression of *Ido1* in NT5/*Ido1*+ cells stimulated tumor cell proliferation.

3.4. Ido1 and Cell Cycle Gene Expression. To further analyze the differences of the cell cycle regulation in *Ido1*+ and *Ido1*− cells, the cell cycle genes expressions were investigated (Table 2). We used the Mouse Cell Cycle PCR Array from SABiosciences to compare cell cycle gene expression in two pairs of positive and negative *Ido1* tumor cells.

Ido1 inhibition in 4T1 tumor cells was associated with significant changes in the expression of 40 cell cycle genes involved in the G1/S transition, S phase, G2/M transition, checkpoints, and M phase, and the majority of the altered genes were downregulated. *Ido1* expression in NT-5/*Ido1*+

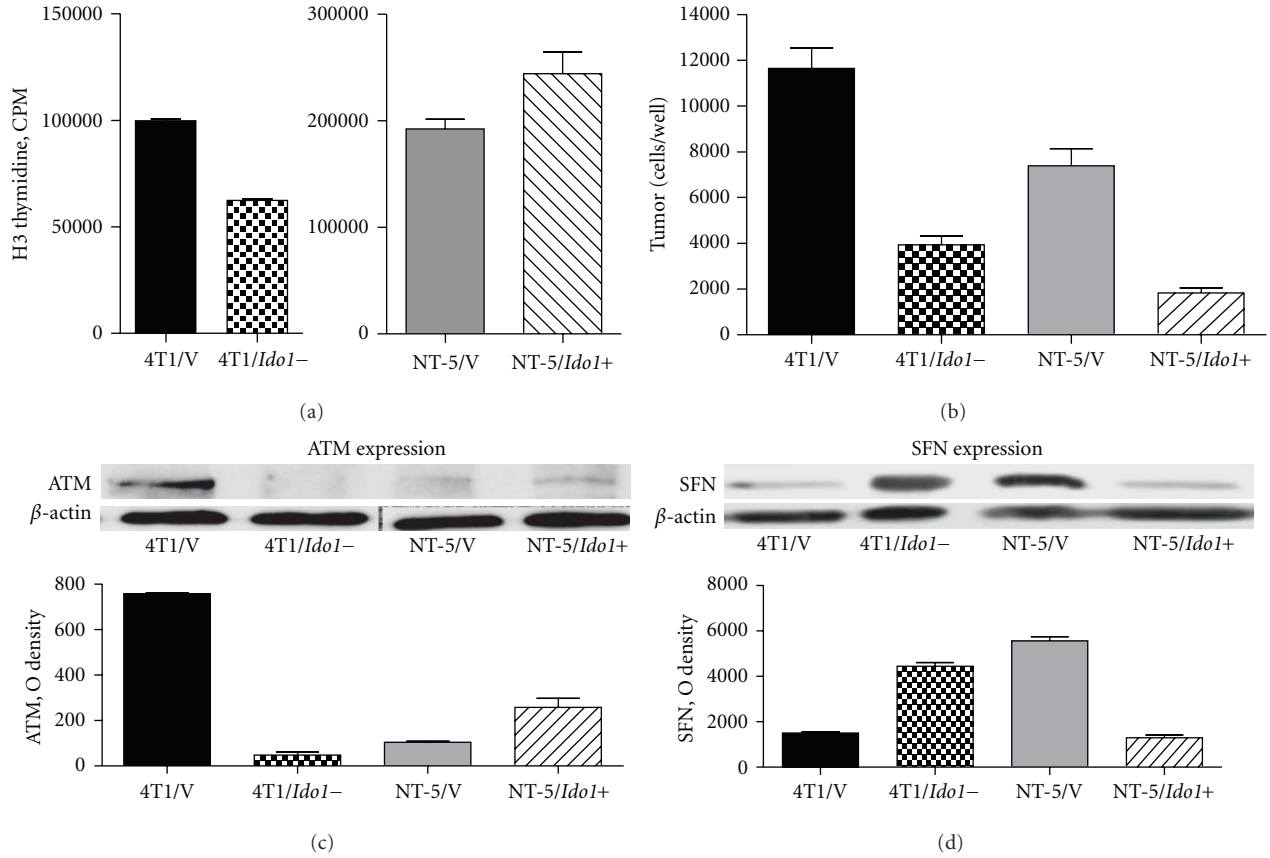

Figure 4: *In vitro* proliferation and western blot analysis ATM and SFN proteins in *Ido1*+ and *Ido1*− breast tumor cells. (a) Analysis of ^{3}H thymidine incorporation and (b) cell counting using Cellomics Array Scan in 4T1/vector, 4T1/*Ido1*−, NT-5/vector, and NT-5/*Ido1*+ cells. (c) Western blots analysis of ATM and (d) SFN (14-3-3 sigma protein) expression in 4T1/vector, 4T1/*Ido1*−, NT-5/vector, and NT-5/*Ido1*+ cells.

cells correlated with changes in the expression of 14 genes, 13 of which were upregulated (Table 2). The expression of four genes (Trp63(p63), dystonin (Dst), stromal antigen1 (Stag1) and microtubule-actin crosslinking factor (Macf1)) was lower in *Ido1* downregulated cells, and higher with *Ido1* upregulation (Table 2). To verify whether the changes in gene expression are associated with upregulation of IDO1 protein expression in *Ido1*+ and *Ido1*− cells, we performed a western blot analysis of the cell cycle regulators ATM and stratifin (Sfn) [35, 36].

Atm (ataxia telangiectasia mutated) is an important cell cycle regulation gene encoding a serine/threonine protein kinase that is recruited and activated by DNA double-strand breaks. ATM phosphorylates several key proteins that initiate activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair, or apoptosis [35, 36]. *Atm* expression was significantly decreased (−18.3-fold) in 4T1/*Ido1*− cells and only slightly changed (1.7-fold) in NT-5/*Ido1*+ (Table 2). As demonstrated in Figure 4(c), ATM is expressed in 4T1/vector (*Ido1*+) *cells,* and ATM production is dramatically reduced in *Ido1*− cells.

Stratifin (Sfn), also named 14-3-3 sigma, is a multifunctional protein involved in cell cycle regulation [36]. Downregulation of *Ido1* in 4T1 cells is associated with upregulation of Sfn gene expression (12 fold); and upregulation of *Ido1* in NT5 cells is associated with a decreased level (−7 fold) of Sfn gene expression (Table 2).

Western blot analysis demonstrated that SFN protein is increased in *Ido1*− cells, and it is reduced in *Ido1*+ cells (Figure 4(d)). Thus, the results of the PCR analysis of *Atm* and *Sfn* gene expression are consistent with the results of the western blot analysis of the ATM and SFN proteins

4. Discussion

We first analyzed the impact of *Ido1* expression on tumor growth and spontaneous pulmonary metastasis formation and then investigated the IDO1-regulated mechanisms involved in tumorigenesis. One of the key experimental strategies to elucidate the function of a gene *in vitro* and *in vivo* is the specific inhibition or upregulation of its expression. Here, we explored both approaches. *Ido1*+ cells were transected with plasmid expressing shRNA for *Ido1,* and *Ido1*− cells were transfected with *Ido1* cDNA. These two pairs of *Ido*+ and *Ido*− clones were evaluated throughout our investigation.

We found that 4T1/*Ido1*+ tumors grew faster than 4T1/*Ido1*− tumors in immunocompetent mice. Growth

Table 2: *Ido1* and the cell cycle genes expression in mouse breast tumor cells.

		Fold changes in cell cycle genes expression	
		4T1/*Ido1*+ versus 4T1/*Ido1*−	NT-5/*Ido1*− versus NT-5/*Ido1*+
		G1 phase and G1/S transition	
1	CAMK2a	1.3	5.4
2	GPR132	4.8	1.8s
3	ITGB1	**−17.6**	1.8
4	PPP2r3a	5.1	2.9
5	PPP3ca	**−4.2**	2.1
6	SKP2	**−3.8**	1.5
		S phase and DNA replication	
7	DNAJC2	7.7	1.5
8	MKI67	8.8	2.3
9	MRE11a	**−4.9**	1.2
10	MSH2	**−3.3**	1.5
11	PCNA	**−5.4**	1.1
12	RAD17	**−6.1**	1.8
13	RAD51	**−5.2**	1.3
		M phase	
14	BRCA2	**−7.1**	1.8
15	CCNA1	19.1	4.4
16	CCNB1	**−10.9**	**−6.8**
17	CDC25a	**−3.1**	1.9
18	NEK2	**−4.4**	1.1
19	NPM2	**−1.7**	3.1
20	PRM1	10.0	1.6
21	RAD21	3.3	2.8
22	SMC1a	8.6	1.4
23	STAG1	**−5.8**	3.0
		G2 phase and G2/M transition	
24	CHEK1	**−31.2**	1.3
25	DNAJC2	**−7.7**	1.5
		Cell cycle checkpoint; cell cycle arrest	
26	BRCA2	**−7.1**	1.8
27	CDK5rap1	**−6.5**	1.2
28	CDKN1a(p21)	11.6	2.0
29	CDKN1b(p27)	**−6.8**	1.0
30	CDKN2a(p16)	9.9	2.4
31	CASP3	**−5.6**	1.4
32	CHEK1	**−31.2**	1.3
33	DDIT3	**−1.8**	14.3
34	DST	**−11.2**	7.3
35	HUS1	**−7.7**	1.0
36	INHA	10.0	5.0
37	MACF1	**−8.7**	3.1
38	NOTCH2	**−1.8**	3.1
39	PKD1	**−2.0**	3.5
40	PMP22	**−1.9**	6.1

Table 2: Continued.

		Fold changes in cell cycle genes expression	
		4T1/*Ido1*+ versus 4T1/*Ido1*−	NT-5/*Ido1*− versus NT-5/*Ido1*+
41	SFN	12.0	**−7.0**
	Regulation of the cell cycle		
42	ABL1	**−3.4**	1.3
43	ATM	**−18.3**	1.7
44	BRCA1	**−12.5**	1.3
45	CCNC	**−11.0**	1.0
46	PBL1	**−8.9**	1.5
47	E2F3	3.3	**−1.2**
48	TRP63(p63)	**−27.3**	6.8

4T1: stable clone with shRNA-negative control vector pGeneClip/Neomycin; 4T1/*Ido1*−: stable clone with shRNA for IDO.
NT-5: stable clone with control vector pRc/CMV/Neomycin; NT-5/*Ido1*+: stable clone with cDNA IDO.
*A positive value (normal font) indicates fold of gene upregulation; a negative value (bold) indicates fold of gene downregulation. Genes which were found up/downregulated more than 3-fold at least in the one of the cell line are presented.

inhibition of *Ido1*− tumor cells can be attributed to a more efficient immune response. This conclusion is based on the following. (1) Spleen cells from mice bearing 4T1/*Ido1*− tumors generated a higher level of cytotoxic T cells (CTCs). These CTCs were able to destroy 4T1/*Ido1*− cells and 4T1/*Ido1*+ cells. However, their cytotoxic effects were higher against 4T1/*Ido1*− cells, most likely due to a higher sensitivity of these cells to apoptotic signaling. (2) In the absence of major immune mechanisms in SCID/beige mice, the growth of *Ido1*− tumors was accelerated, suggesting a role for the immune system in *Ido1*-dependent control of tumor growth. These findings are consistent with previous publications, demonstrating the role of IDO1 in tumor immunosuppression [5–9, 12–14, 37].

Activation of *Ido1* by IFN-γ links inflammation and cancer immune surveillance [11, 28, 35], and an increase in IFN-γ within the tumor microenvironment also induced IDO1 expression in APCs and DCs that localize to the tumor-draining lymph nodes [38]. We found that IFN-γ treatment induced *Ido1* expression in *Ido1*+ cells and had no effect on *Ido1*− cells. Recently, a mouse genetic study revealed a critical role for IDO1 in supporting inflammatory skin carcinogenesis [26]. An analysis of IDO1 dysregulation in cancer showed that JAK/STAT and NF-κB signaling are also essential for IDO1 induction in oncogenically transformed skin epithelial cells [39]. The anti-inflammatory agent, ethyl pyruvate, was found to be able to block *Ido1* expression, indicating a link between IDO1 and inflammatory processes [39].

For the first time, we showed that *Ido1* regulates tumor metastasis via immunosuppressive mechanisms. *Ido1*+ tumors develop dramatically higher levels of spontaneous pulmonary metastasis in immunocompetent mice than *Ido1*− tumors. Levels of lung metastasis in SCID/beige mice (deficient in T cells, B cells, and NK cells) are higher than in immunocompetent mice, thus confirming an immunosuppressive role for IDO1 in spontaneous metastasis formation.

In contrast to our expectation, growth of the *Ido1*− tumors was slower than growth of *Ido1*+ tumors in immunodeficient SCID/beige mice, suggesting that some *Ido1*-associated nonimmunological mechanisms may be involved in tumor cell growth regulation. It is conceivable that *Ido1*− cells may have an increased sensitivity to apoptosis signaling or slower cell cycle progression and proliferation than *Ido1*+ cells. Our *in vitro* study revealed multiple differences between *Ido1*− *and Ido1*+ cell populations suggesting the complexity of IDO1- regulated tumor cell functions. *Ido1*− cells grow slower than *Ido1*+ cells. Similarly, the inhibition of IDO expression in rat NMU-induced breast cancer showed the reduced cell proliferation *in vitro* [40].

Ido1+ cells have small proportions of apoptotic cells in normal media and in starved conditions, and they recover better after starvation. In contrast, *4T1/Ido1*− cells, in which the *Ido1* gene was down-regulated via shRNA transfection, have dramatically increased sensitivity to apoptosis, thus suggesting an important antiapoptotic function of *Ido1* in tumor cells. However, molecular pathways involved in *Ido1*-associated apoptosis signaling remain virtually unexplored, offering new areas for investigation.

Cell cycle distributions within *Ido1*− and *Ido1*+ tumor cell populations also differ in that *Ido1*+ cells show a higher proportion of cells in S and M phases than *Ido1*− cells. Using a comparative RT-PCR analysis of cell cycle regulatory gene expression, we found that downregulation of *Ido1* is associated with decreased expression of multiple genes involved in regulation of G1/S transition, S phase, G2/M transition, checkpoints, and M phase, whereas upregulation of *Ido1* induces expression of these genes. Protein analysis of the products of two genes, stratifin (*SFN*) and *ATM*, in *Ido1*+ and *Ido1*− cells show that the up- and downregulation of these genes correlates with the increased/decreased production of their respective proteins. Thus, the roles of *Ido1* in breast tumor growth are complex and suggest the involvement of additional nonimmunological mechanisms.

5. Conclusion

Our primary finding is that *Ido1* expression has immunological and nonimmunological effects on breast tumor

growth and spontaneous pulmonary metastasis formation. We demonstrated that IDO1 is involved in the regulation of tumor cell proliferation *in vitro* and *in vivo*. *Ido1* expression also affects apoptosis in tumor cells. The involvement of IDO1 in cell cycle regulation and tumor cell stress response, in addition to the known role of IDO1 in tumor immunosuppression, make it an attractive target for antitumor therapy.

Authors' Contribution

V. Levina and Y. Su have equally contributed to this work.

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Review Article

Programmed Death Ligand 2 in Cancer-Induced Immune Suppression

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Inhibitory molecules of the B7/CD28 family play a key role in the induction of immune tolerance in the tumor microenvironment. The programmed death-1 receptor (PD-1), with its ligands PD-L1 and PD-L2, constitutes an important member of these inhibitory pathways. The relevance of the PD-1/PD-L1 pathway in cancer has been extensively studied and therapeutic approaches targeting PD-1 and PD-L1 have been developed and are undergoing human clinical testing. However, PD-L2 has not received as much attention and its role in modulating tumor immunity is less clear. Here, we review the literature on the immunobiology of PD-L2, particularly on its possible roles in cancer-induced immune suppression and we discuss the results of recent studies targeting PD-L2 in cancer.

1. Introduction

Molecules of the B7-CD28 family play an important role in T-cell activation and tolerance. These pathways are not only responsible for providing positive costimulatory signals to sustain T-cell activity, but also contribute inhibitory signals that modulate the magnitude of T-cell responses [1]. Useful as this negative feedback may be during physiological homeostasis, it may be a problem in the context of cancer. It is now clear that the inhibitory members of the B7-CD28 family are upregulated by a variety of cells within the tumor microenvironment [2]. Thus, the selective blockade of these inhibitory molecules is an attractive approach to cancer immunotherapy.

The programmed death-1 receptor (PD-1, CD279) with its ligands PD-L1 (CD274, B7-H1) and PD-L2 (CD273, B7-DC) constitutes one such inhibitory pathway. Therapeutic antibodies for blocking PD-1 and PD-L1 have been developed and are undergoing human clinical testing [3, 4]. Negating the PD-1/PD-L1 interaction is of particular interest as PD-L1 is upregulated by many human cancers [5]. On the other hand, the role of PD-L2 in modulating immune responses is less clear, and its expression is more restricted compared to PD-L1, thus making it a less obvious target in cancer immunotherapy. However, in this context, several aspects of PD-L2 biology deserve attention, including a partial contextual dependency of PD-L2 expression. Recent reviews have discussed the importance of PD-L1 in tumor immunology [4, 6]. Here, we will focus on the immunobiology of PD-L2 and particularly on its possible roles in cancer-induced immune suppression.

2. Expression Pattern of PD-L2

The patterns of expression of PD-L1 and PD-L2 are quite distinct. PD-L1 is constitutively expressed by a wide variety of immune cells and nonimmune cells and most normal tissue cells seem to be able to upregulate PD-L1 in

the presence of strong inflammatory signals, presumably to prevent collateral damage induced by potent but potentially destructive Th1/17 T-cell responses [7–10]. Compared to PD-L1, constitutive basal expression of PD-L2 is low. PD-L2 expression was initially thought to be restricted to antigen-presenting cells such as macrophages and dendritic cells (DCs) [11]. In recent years however, several groups have shown that PD-L2 expression can be induced on a wide variety of other immune cells and nonimmune cells depending on microenvironmental stimuli [12–17].

Exposure of DCs and macrophages to Th2 (IL-4) cytokines increased the expression of PD-L2 as did IFNγ and toll-like receptor ligands [17, 18] (Figure 1). In addition, cytokines binding to receptors that use the common γ-chain such as IL-2, IL-7, IL-15, and IL-21 upregulated PD-L2 in these cells [12]. Alveolar epithelial cells express high levels of PD-L2 in the presence of IL-4 when infected with respiratory syncytial virus [10]. Constitutive expression of PD-L2 on human umbilical vein endothelial cells has been observed and stimulation by IFNγ and TNFα *in vitro* further enhanced its expression [19]. Also human colonic fibroblasts have been shown to express PD-L2, resulting in T-cell suppression in the gut epithelial mucosa [9]. Of special importance to the field of tumor immunology is the finding that not only normal fibroblasts, but also cancer-associated fibroblasts can constitutively express PD-L2 (further discussed below) [20]. Recently, constitutive expression of PD-L2 was found on 50–70% of mouse peritoneal CD5$^+$ B cells and PD-L2 expression was found to be unique to this particular subset of B cells [13]. An additional level of complexity was discovered in the finding that T cells themselves can upregulate PD-L2 upon activation *in vitro* [21, 22]. We have shown that this was predominantly the case for Th2 cells activated in the presence of IL-4, and less so for Treg, Th1, and Th17 cells [23]. From these data a new picture is emerging in which the expression of PD-L2 is much less restricted than previously thought and at least for some cells partly depends on microenvironmental cues, with a specific role for Th2 cytokines.

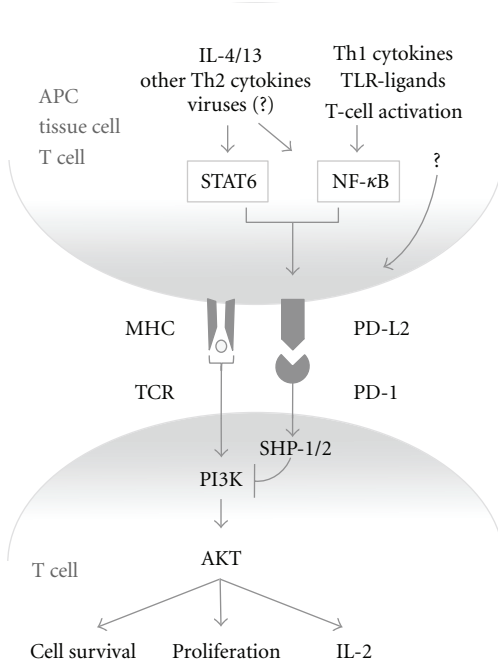

Figure 1: PD-L2/PD-1 signaling. PD-L2 expression by different cell types is regulated by STAT6 and NF-κB, although other possible regulators cannot be excluded. The most potent inducers of PD-L2 expression appear to be Th2 cytokines, particularly IL-4. Several new activators of STAT6 (such as viruses) have been found, but whether they therefore also upregulate PD-L2 is not known yet. PD-L2/PD-1 interaction results in the suppression of TCR-induced PI3K/AKT activation and subsequent attenuation of T cell survival, cytokine production and proliferation.

3. Regulation of PD-L2 Expression

From the data discussed above, it can be inferred that signalling pathways downstream of cytokine receptors and innate immune activators play an important role in the regulation of PD-L2 expression. Indeed, two major pathways that have been reported to regulate PD-L2 expression are the NFκB-pathway and the signal transducer and activator of transcription (STAT) 6 pathway (Figure 1). Two groups have found that macrophages from Stat6$^{-/-}$ mice are unable to express PD-L2 [24, 25]. These results were confirmed in bone-marrow-derived DCs from Stat6$^{-/-}$ mice and in human monocyte-derived DCs in which STAT6 was pharmacologically inhibited [17]. STAT6 is a signaling molecule and transcription factor that is especially important in the regulation of Th2 immune responses and it is activated by ligation of the IL-4 and IL-13 receptor with its ligands IL-4 or IL-13 [26]. Recently, also the cytokines TSLP and IL-27 have been shown to activate STAT6, as well as viruses in a JAK-independent manner, providing the possibility that these stimuli may also induce PD-L2 expression [27–29].

NFκB was shown to play a role in the regulation of PD-L2 expression by Liang and colleagues: although knockdown of NFκB did not completely abrogate PD-L2 expression, DCs from NF-κB p50$^{-/-}$ p65$^{-/+}$ mice had lower levels of expression and were less able to upregulate PD-L2 when stimulated with exogenous IFNγ or LPS [30]. However, NF-κB p50$^{-/-}$ mice are severely hampered in the production of the STAT6 activating cytokines IL-4 and IL-13 [31], possibly explaining the lowered PD-L2 expression found by Liang and colleagues. Indeed, in contrast to the findings by Liang et al., another study found that the PD-L2 promoter could be activated by IL-4 signaling but not by LPS signaling, a strong NF-κB inducer [32]. Thus, it seems that NF-κB does not play a direct role in the induction of PD-L2 expression. However, an indirect role cannot be ruled out since at least one study showed that pharmacological blocking of NF-κB interfered with STAT6 DNA binding but not phosphorylation or nuclear translocation, indicating that

NF-κB might have a role in regulating STAT6 DNA binding activity and thus indirectly controls PD-L2 expression [33]. Together, these findings hint at the possibility that PD-L2 may be of particular importance in the regulation of Th2 type immune responses. Whether the NFκB and STAT6 pathways are the only pathways that are of importance for the regulation of PD-L2 expression remains an open question.

4. Molecular Consequences of PD-L2/PD-1 Interactions

The structures of PD-1/PD-L1 [34] and PD-1/PD-L2 [35] reveal differences in the binding modalities, which helps explain the distinct molecular mechanisms of interaction between PD-1 and its ligands. By investigating PD-1 interactions with its ligands by surface plasmon resonance and cell surface binding, Ghiotto et al. showed that while PD-L2 interact in a direct manner with PD-1, PD-L1 binding to PD-1 involves complex conformational changes. The notion of PD-L1 and PD-L2 simultaneously binding to PD-1 was also dispelled, indicating that the two ligands cross-compete to bind to the receptor [36]. Furthermore, the relative affinity of PD-L2 to PD-1 was calculated to be 2–6-fold higher than that of PD-L1 [37]. If expressed at the same level, PD-L2 would be expected to outcompete PD-L1 for binding PD-1, but the physiological relevance of this competition is not yet fully clear [38]. The fact that PD-L2 is generally expressed at a lower level may favour PD-L1 as the primary binding ligand of PD-1, except for during Th2 responses when PD-L2 is upregulated.

PD-1/PD-L interactions lead to phosphorylation of two tyrosines at the intracellular tail of PD-1. These tyrosines are part of an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) and an Immunoreceptor Tyrosine-based Switch Motif (ITSM). ITSM then recruits either of two structurally related protein tyrosine phosphatases, SH2-domain containing tyrosine phosphatase 1 (SHP-1) and SHP-2 [39], which suppress activation of PI3K/Akt [40]. Consequently, the survival factor Bcl-xL is downregulated [40] and expression of transcription factors associated with effector cell function including GATA-3, T-bet and Eomes are lost [41]. The net result of these PD-1-induced cascades is an impairment of proliferation, cytokine production, cytolytic function, and survival of the T cell [42].

Whether PD-L2 can induce signalling downstream of its intracellular domain has not been well characterized. Using magnetic beads coated with anti-CD3 and anti-CD28 as artificial antigen-presenting cells, Messal and colleagues found that when PD-L2 on T cells was ligated with the same beads coated with anti-PD-L2, T-cell proliferation and production of IL-2, IL-10, and IFNγ was significantly decreased [21]. Studies from a different group demonstrated that cross-linking of PD-L2 on T cells that were transduced with PD-L2 siRNA resulted in the elimination of the negative effect on IFNγ production [22]. These data indicate that indeed PD-L2 does induce signalling downstream and as such plays a role in the modulation of T-cell function, but the exact molecular pathway is yet to be elucidated.

Of note, PD-1 may not be the only receptor for PD-L2. This can be inferred from helminth infection and allergic animal models, showing enhanced disease severity when PD-L2 blocking antibodies were used, but not when PD-1 blocking antibodies were used [43, 44]. Furthermore, PD-L2 mutants with abolished PD-1 binding capacity could still exert functional effects on T cells from normal and PD-1-deficient mice [45]. Thus, although for PD-L1 another receptor has been found in B7.1, for PD-L2 this still remains enigmatic [46].

5. Physiological Function of PD-L2

The initial finding of enhanced expression of PD-L2 on activated professional antigen-presenting cells suggested that PD-L2 mainly functioned in the induction phase of T-cell immunity, whereas PD-L1 which is much more widely expressed, played an important role in constraining activated T cells at the effector site. However, the above-mentioned data showing a wider inducible expression of PD-L2 as well as *in vivo* animal studies have demonstrated that PD-L2 probably functions both at the induction phase as well as the effector phase of T-cell responses. For example, antigen-presenting cells from PD-L2$^{-/-}$ mice displayed an enhanced T-cell activating potential both *in vitro* and *in vivo* [47]. Inducible experimental autoimmune encephalitis models have shown that therapeutic blockade of PD-L2 results in enhanced disease severity not only when the antibodies were administered at the time of disease initiation, but also in the chronic phase [48, 49].

The physiological role of dampening and regulating T-cell responsiveness seems especially important in the mucosal immune response against environmental antigens [50]. For example, in PD-L2$^{-/-}$ mice experimentally induced oral tolerance to chicken ovalbumin was abrogated, and animal models using exposure to environmental allergens through the lung mucosa demonstrate enhanced airway hypersensitivity when PD-L2 (but not PD-L1) is knocked out or pharmacologically blocked [43, 47, 51]. Experimental colitis models, however, have thus far not demonstrated a role for PD-L2 in controlling disease severity [52]. A possible explanation for this lack of effect could be that the colitis induction in these models (adoptive transfer of CD4^{+}CD45RB^{high} T cells into SCID mice) probably does not involve a Th2-skewed microenvironment. Although immune infiltrates in human ulcerative colitis have been shown to highly express PD-L1 [52], this has not been investigated for PD-L2.

As can be inferred from the above-mentioned asthma models, as well as from helmintic infection animal models demonstrating enhanced disease severity in the absence of PD-L2 signalling [44], PD-L2 appears to predominantly be of significance in the modulation of Th2 immune responses. Animal models of Th1-driven diseases, without a dominant Th2 component, such as autoimmune diabetes have generally shown a more dominant role of PD-L1 over PD-L2 in restraining T-cell activity and prevention of subsequent collateral tissue damage [53, 54].

Intriguingly, in a PD-L2 knock out mouse model different from the previously discussed ones [47, 51], IFNγ-production by T-helper cells as well as IFNγ-dependent humoral responses and antigen-specific CTL responses were impaired, indicating that PD-L2 also functions as a tuning molecule that can even augment CTL and Th1 responses [55]. However, using an *in vitro* system of engineered T-cell stimulator cells that detached PD-L2/PD-1 interactions from the context of other molecules regulating T-cell activation, no positive costimulatory role for PD-L2 was found [56].

Although the final verdict is still out, and PD-L1 and 2 do appear to have overlapping effects, together these data indicate that the main physiological function of PD-L2 could lie in the dampening and regulation of Th2-driven T-cell immune responses both during the induction and the effector phase, with possibly special significance in mucosal responses against environmental antigens. However, given the fact that PD-L2 also inhibits IFNγ production by Th1 cells, and Th2 responses appear to prevent acute tissue damage by Th1 or Th17 cells, as has been shown very recently in a helminth model [57], it could be hypothesized that although the Th2 response is the "driver" of PD-L2 expression, the potentially destructive Th1/17 component of the local immune response is the eventual target.

6. PD-L2 in Cancer

Since PD-L2 appears to play an important role in the modulation of Th2 responses, while in the context of antitumor immunity Th1 responses are the most potent, it does not seem obvious to choose PD-L2 as a target in cancer. However, in recent years evidence has accumulated showing that tumor microenvironments are often deviated towards an ineffective Th2 type of immune milieu, resulting in cancer cell escape from immune surveillance. For example, breast cancer cells have been shown to produce IL-13 themselves, resulting not only in autocrine STAT6-phosphorylation but also in the instruction of DCs to skew CD4 T cells towards a Th2 phenotype with high production of IL-4 and IL-13 [58]. In addition, human and murine studies in pancreatic cancer have shown high local production of TSLP (another STAT6-activating cytokine [27]), resulting in Th2 skewing and enhanced tumor outgrowth [59, 60]. PD-L2 upregulation in response to local Th2 cytokines may thus affect tumor-specific CTL reactivity, either in the induction phase in the tumor-draining lymph node or in the effector phase in the tumor. Hence, there is a clear rationale to further investigate the relevance of PD-L2 in cancer.

6.1. Clinical Relevance of PD-L2 Expression in Cancer. Given the possible immune evasion to antigen-specific T cells by PD-L2-expressing tumor cells, several groups have investigated the possible correlation between tumor PD-L expression and clinical outcome in retrospective patient cohorts. These studies were performed before the observation was made that also cancer-associated fibroblasts upregulate both PD-L1 and 2 [20], and therefore a clear distinction between tumor cell and tumor stroma expression may not have been made. Ohigashi et al. [61] investigated the expression of PD-L1 and PD-L2 in human esophageal cancer to determine their clinical significance in patients prognosis after surgery. Using RT-qPCR and immunohistochemistry, the authors showed that both PD-L1 and PD-L2 are expressed in frozen tissue samples of esophageal cancer patients and PD-L2-positive patients had a poorer prognosis than the negative patients, as was the case for PD-L1 [61]. Interestingly, there was a significant inverse correlation between PD-L2 expression and CD8 TILs but not CD4 TILs. In a retrospective study involving 51 patients with pancreatic cancer, 27% of the analyzed tumors expressed PD-L2 versus 39% expressing PD-L1. No correlation was found between PD-L2 expression and survival, whereas PD-L1 expression correlated with an impaired survival [62]. Similarly, in a cohort of 70 patients with ovarian cancer, the majority of the tumors were negative or weakly positive and although PD-L2 expression was correlated with an impaired survival, this did not reach statistical significance [63]. And lastly, in a study involving 125 patients with hepatocellular carcinoma a minority had high PD-L2 expression, and again, although PD-L2 expression was correlated with an impaired disease-free survival, this difference was not statistically significant [64].

Thus, the majority of studies have found a significant correlation between impaired survival and PD-L1 expression, but much less so for PD-L2. Although several studies have found an impaired survival in patients with PD-L2 expressing tumors, this reached statistical difference in only one of these studies [61]. However, it is important to note that in the majority of studies PD-L2 was expressed in only a minority of patients. In addition, it is not inconceivable that PD-L2 expression is more dependent on environmental cues than PD-L1, which seems to be expressed in a more constitutive manner, although this can be further upregulated with proinflammatory stimuli [65]. In fact, if the PD-L's are induced in response to IFNγ that is produced by antigen-specific tumor-infiltrating T cells, a process recently termed adaptive resistance [4], this may actually reflect a positive event in the context of antitumor immunity, but this does make the data more difficult to interpret. Finally, there are some technical issues with different findings depending on whether frozen sections or paraffin-embedded slides were used, with a higher percentage of positive tumors from frozen sections, as has been shown for PD-L1 [66]. Thus for these reasons, although PD-L1 may indeed be the more dominant negative inhibitory molecule in the context of tumor immunology, PD-L2 should not yet be dismissed as a possible second important suppressive molecule in the tumor microenvironment.

It is also important to note here that perhaps not only PD-L2 expression by the tumor cells themselves, but rather by stromal cells is of importance. Nazareth and colleagues found constitutively high PD-L1 and 2 expression in fibroblasts that were cultured from human non-small-cell lung cancers [20]. This expression appeared to be functional, since *in vitro* blocking studies demonstrated that the fibroblasts inhibited IFNγ-production by autologous T

cells in a PD-L1- and 2-dependent manner. For this reason, future studies should not only focus on PD-L expression by tumor cells only, but also by the tumor stroma.

6.2. Therapeutic Studies Targeting PD-L2 in Cancer. Given its potential role in cancer-associated immune suppression in the tumor microenvironment, targeting the PD-1/PD-L pathway seems an attractive treatment strategy. Several studies have investigated the therapeutic effect of blocking antibodies against the PD-1/PD-L pathway in murine cancer models, demonstrating enhanced tumor control rates, though in none of these studies the blocking of PD-L2 was used as a defined treatment strategy [67–70]. Although in a few studies PD-L2 blocking strategies were used, this was always in combination with the targeting of PD-L1 [71, 72]. In these studies again impaired tumor outgrowth was demonstrated. The true additive value of adding anti-PD-L2 on top of anti-PD-L1 cannot be assessed based on these studies, since separate single-antibody treatments were not tested.

In one study using the Panc02 murine pancreatic tumor model, decreased tumor outgrowth rates on day 21 were seen when the animals were treated with PD-L2 blocking antibodies, comparable to that seen with blocking PD-L1 or PD-1 alone [73]. In contrast with these data, in a hepatic metastasis model of CT-26 colon cancer, PD-L2$^{-/-}$ mice displayed impaired survival and increased tumor outgrowth in combination with a decreased tumor-specific CTL response [55]. It is difficult to reconcile these conflicting data, but the difference in outcome may be the result of a difference in mouse strain backgrounds or differences in the local tumor microenvironment and cytokine milieu that influence PD-L2 expression by its several constituent cell types.

Human data about targeting PD-L2 in cancer are scarce. Currently a phase I study is ongoing investigating AMP-224, a recombinant fusion protein of PD-L2 and the Fc portion of IgG1 (http://ClinicalTrials.gov Identifier NCT01352884) [74]. Although there are no results to date about specifically targeting PD-L2 in humans, promising results have been seen with antibodies targeting PD-1 with objective responses in several types of cancer and with tolerable toxicity, specifically autoimmune-related adverse events [3, 4]. In addition, several groups have used approaches other then antibodies to target PD-L2 in humans. Hobo and colleagues used siRNA to knock down PD-L1 and PD-L2 in DCs, with the ultimate goal of incorporating this approach in DC-based cancer vaccines. PD-L2-silenced DCs modestly improved IFNγ production by allogeneic T cells, but double knockdown of both PD-Ls resulted in a synergistic increase of IFNγ production and proliferation capacity of antigen-specific T cells *in vitro* [75]. This was also followed by a synergistic improvement of cytokine production in double PD-L blockade compared to single PD-L1 knockdown or PD-L2 knockdown [75].

Recently, we found that platinum-based chemotherapeutic drugs that form the cornerstone in the medical treatment of many cancers, dephosphorylate STAT6, resulting in downregulation of PD-L2 by DCs [17]. We found that this resulted in an enhanced T-cell activating potential of the DCs in vitro. Moreover, also tumor cells downregulated PD-L2 when treated with platinum drugs, resulting in enhanced CTL recognition. Indeed tumor STAT6 expression correlated strongly with an enhanced recurrence-free survival in a cohort of patients with head and neck cancer that had been treated with cisplatin-based chemoradiation. Conversely, in a cohort of patients that had been treated with radiotherapy alone, STAT6 expression showed a clear trend towards a poor clinical outcome, which could be explained by the immune-evasive potential of STAT6-expressing tumor cells, if not attacked by platinum. Although in this study it could not be ruled out that STAT6-dependent effects other than PD-L2 upregulation also played a role, these results indicate that we may in fact already be targeting PD-L2 in cancer patients with one of the clinically most widely used groups of chemotherapeutics [76].

However, to truly determine whether PD-L2 is a relevant molecule to target in cancer immunotherapy, more studies are necessary. Given the dependency of PD-L2 expression on environmental cues, the outcomes may differ between tumor models and tumor types in animals and humans, or even between patients with the same tumor type. Future studies should investigate whether it is possible to predict which patients might respond to PD-L2 blockage by first defining the type of immune response occurring in the tissue, for example, whether it is Th2 or not. In addition, double blockade combining PD-L1 and 2, or combining anti-PD-L2 with anti-CTLA4, which blocks an immune checkpoint more during the induction phase could potentially be more efficient [67]. Finally, since several forms of cancer chemotherapy have been shown not only to induce antigen release but also subsequent immune activation [77, 78], the therapeutic efficacy of these drugs could potentially be further enhanced by combining it with PD-L2 blockade.

7. Conclusion

It has now been demonstrated that PD-L2 is principally an inhibitory molecule, expressed not only by antigen-presenting cells, but also by other immune cells including T cells themselves and nonimmune cells in an inducible manner, mainly through Th2-associated cytokines. Based on the current literature, it is not yet possible to draw a definite conclusion on the relevance of PD-L2 in the immune-suppressive tumor microenvironment, although there are some encouraging data indicating that targeting PD-L2 in cancer may be a viable treatment approach. Therefore, more studies targeting PD-L2 in the context of antitumor immunity are urgently needed.

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Review Article

Releasing the Brake: Targeting Cbl-b to Enhance Lymphocyte Effector Functions

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The E3 ubiquitin ligase Cbl-b is an established nonredundant negative regulator of T-cell activation. Cbl-b fine-tunes the activation threshold of T cells and uncouples T cells from their vital need of a costimulatory signal to mount a productive immune response. Accordingly, mice deficient in *cblb* are prone to autoimmunity and reject tumors. The latter has been described to be mediated *via* $CD8^+$ T cells, which are hyperactive and more abundant in shrinking tumors of *cblb*-deficient animals. This might at least also in part be mediated by resistance of *cblb*-deficient T cells to negative cues exerted by tumor-associated immuno-suppressive factors, such as TGF-β and regulatory T cells (Treg). Experiments using *cblb*-deficient T cells either alone or in combination with vaccines validate the therapeutic concept of enhancing the efficacy of adoptively transferred lymphocytes to treat malignant tumors. This paper summarizes the current knowledge about the negative regulatory role of Cbl-b in T-cell activation and its potential therapeutic implications for cancer immunotherapy.

1. Introduction

Maintenance of tolerance and induction of T-cell anergy is critical for prevention of autoimmunity. However, in the case of malignancies, tumor-induced T-cell anergy and/or tolerization induces cancer-associated immune paralysis, which at least in part contributes to uncontrolled tumor growth and metastasis. In 2000, two groups independently described that the E3 ligase Cbl-b functions as a "gate keeper" in peripheral T-cell tolerance [1, 2]. Cbl-b is a member of the highly conserved family of Cbl (casitas b-lineage lymphoma) proteins (Figure 1) and ubiquitinylates substrates by its E3 ligase activity via its RING domain. The name is derived from the retroviral oncoprotein v-Cbl, which promotes development of b-cell lymphoma in mice [3]. Target ubiquitinylation induces either proteasomal or lysosomal degradation regulating protein turnover or modifies the function of target proteins by altered subcellular localization. Cytoplasmic signaling proteins and nuclear transcription factors tend to be polyubiquitinated for subsequent proteasomal degradation, whereas ligand-activated surface receptors, such as receptor tyrosine kinases, G-protein-coupled receptors, and the T-cell receptor (TCR) are more often regulated by endocytosis followed by lysosomal degradation [4–6]. Substrates can either be tagged with single (monoubiquitinylation) or multiple (>4) ubiquitins, the latter leading to polyubiquitinylation. These polyubiquitin chains are generally linked by lysine residues at position 48 or 63. It is known that polyubiquitintagging of substrates induces their degradation by the 26S proteasome, but polyubiquitin chains might also modify protein function, for example, by increased cell-surface-receptor turnover or an altered subcellular localization. In contrast, monoubiquitinylation has been shown to target cell-membrane-receptor-associated proteins to the lysosome, thereby attenuating cell-surface-receptor-mediated signals by a desensitization process. Thus, monoubiquitinylation and K63-linked polyubiquitinylation rather regulate protein trafficking and cell-cell interactions, whereas K48-linked

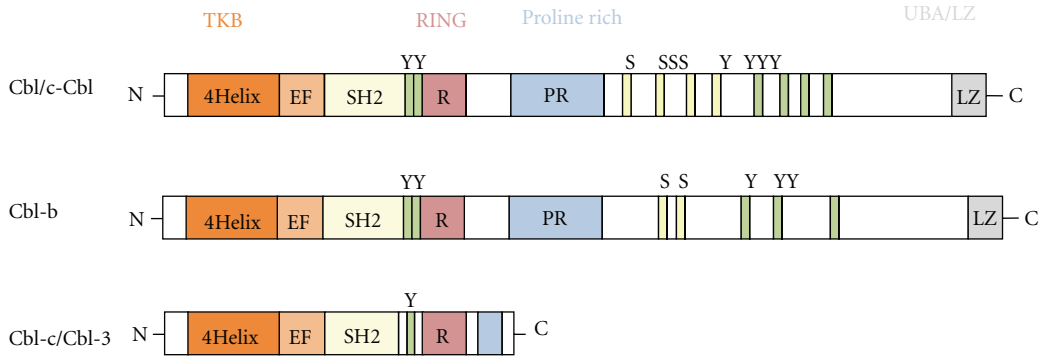

Figure 1: The mammalian Cbl protein family. Schematic representation of the domain architecture of the three mammalian Cbl isoforms, c-Cbl, Cbl-b, and Cbl-c/Cbl-3. The Cbl proteins are highly conserved in the N-terminal region where they comprise a tyrosine-kinase-binding domain (TKB), which is composed of a 4-helix bundle (4H), a calcium-binding EF domain, and a variant SH2 domain that is linked with the RING finger domain. The COOH-terminal region contains proline-rich stretches, multiple serine and tyrosine phosphorylation sites and a ubiquitin-associated UBA domain, and a leucine zipper. The Cbl-c isoform lacks the UBA domain and the leucine zipper domain. TKB, tyrosine-kinase-binding domain; 4H, four-helix bundle; EF, EF hand; SH2, Src-homology domain 2; R, RING "really interesting new gene" finger domain; PR, proline rich domain; Y, tyrosine residue; S, serine residue; LZ/UBA, leucine zipper/ubiquitin associated domain.

polyubiquitinylation targets substrates to the proteasome for degradation [7]. Cbl-b is critically involved in these ubiquitinylation processes, and due to its preferential expression in immune cells it is centrally involved in the regulation of immune responses. In more detail, deficiency of Cbl-b in T-cells induces a prominent hyperactive phenotype, resulting in systemic signs of autoimmunity in animals lacking a functional *cblb* gene [1, 2]. It is tempting to speculate that inhibition of its function might also represent a rational approach to increase T-cell reactivity in the cancer setting. *Vice versa*, in the case of an exaggerated immune response, such as seen in autoimmunity and graft rejections, induction of Cbl-b might be an attractive strategy to limit T-cell reactivity finally inducing tolerance.

This review will summarize our current knowledge on the role of Cbl-b as regulator of T-cell effector function with a particular focus on its potential therapeutic use as target in cancer immunotherapy.

2. Cbl-b is a Member of the Cbl Family of Proteins

Cbl-b is a member of a highly conserved family of Cbl proteins, which in mammals consists of three Cbl genes: Cbl (also termed c-Cbl, Cbl2,or RNF55), Cbl-b (also termed RNF56), and Cbl-c/Cbl-3 (also termed Cbl-SL or RNF57) (Figure 1) [8]. Cbl proteins interact with target proteins via their protein-protein interaction domains, allowing regulation of multiple signaling pathways [9, 10]. The E3 ligase activity of c-Cbl as well as the other Cbl proteins has been shown to depend on the RING-type zinc finger domain [11, 12]. Data from *in vitro* experiments using deletion mutations of c-Cbl highlight the critical role of the RING finger domain for its E3 ligase activity, as RING finger domain mutants could not ubiquitinylate the EGF receptor [13, 14] with the consequence that ligand-induced c-Cbl-mediated desensitization of EGFR receptor expressing cells to EGF was disrupted. The RING finger domain also binds the E2 conjugating enzyme and mediates transfer of ubiquitin between E2 and target substrates [11]. Loss of function mutations in the E3-ligase domain of *cblb* in mice phenocopies the *cblb*-knockout phenotype [15], again supporting the important role of the E3 ubiquitin ligase activity for Cbl-b function.

3. Cbl-b as Master Regulator of T-Cell Effector Functions

Following initial triggering of the antigen receptor, the Src family kinases Lck and Fyn are recruited and phosphorylate Zap-70 (zeta-associated protein of 70 kDa), which initiates various downstream signaling pathways [16]. ZAP-70 phosphorylates SLP-76 mediating formation of a multisubunit protein complex including key signaling molecules such as phosphatidylinositol 3-kinase (PI3K), PLC (phospholipase C), and Vav1. Activation of these signaling components results in the activation of PLCy-regulated Ca^{++} influx, cytoskeletal rearrangements via the nucleotide exchange factor Vav1, Rac,and WASP, and activation of the protein kinase Cθ (PKCθ) [17–19]. The activation step of PKCθ is essential for an appropriate NFκB stimulation to induce a productive T-cell immune response *in vivo*. Of note, PKCθ also functions as critical intermediary for inactivation of Cbl-b in response to costimulation of T-cells through CD28 [20].

In a physiological context, Cbl-b apparently acts as a negative regulator of the T-cell activation process (Figure 2). Accordingly, *cblb*-deficient mice are highly susceptible to spontaneous and antigen-induced experimental autoimmune diseases [1, 2], despite normal thymic T-cell selection and normal peripheral T cell numbers. When isolated, *cblb*-deficient T cells are hyperproliferative and bypass the requirement for CD28 costimulation, that is, they proliferate upon sole stimulation via the TCR comparable to WT T cells stimulated with both anti-CD3 and anti-CD28 stimulating antibodies [1, 2]. Moreover, they produce markedly higher levels of

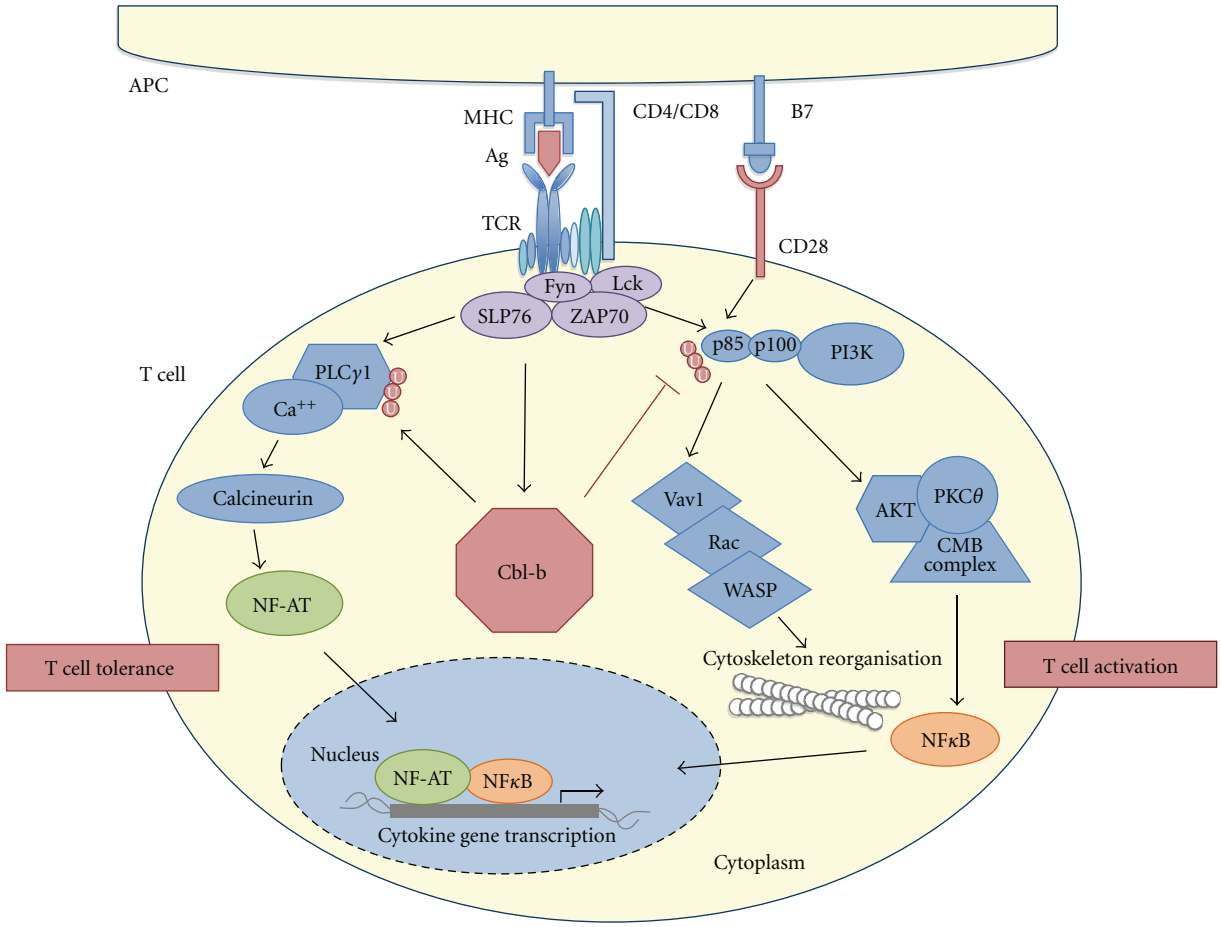

FIGURE 2: Cbl-b functions as central gate keeper of T-cell activation. T-cell stimulation via the TCR and the second costimulatory signal leads to the proximal activation of signaling pathway components. In the absence of costimulation, Cbl-b promotes antigen-specific T-cell tolerance. Thus, Cbl-b functions as negative regulator of the activation of T cells that can be, however, bypassed by CD28 stimulation. In the absence of Cbl-b, T cells are not dependent on a costimulatory signal and proliferative as well as cytokine response upon sole TCR-activation is comparable to WT T cell stimulated via the TCR and CD28. Note that the signaling cascades and interactions are simplified and do not show all molecules involved. For further details please refer to text. Flat-ended lines indicate inhibitory interactions. APC, antigen—presenting cell; U, Ubiquitin.

proinflammatory cytokines, such as IFN-γ [21]. Accordingly, T-cell anergy promoting conditions, such as TCR stimulation in the absence of CD28 costimulation, induce Cbl-b expression [22].

The TCR induces Ca^{++} influx and subsequent activation of the transcription factor NFAT, which leads to activation of the early growth response genes Egr-2 and Egr-3, which then increase Cbl-b expression [4, 23]. Cbl-b in turn regulates recruitment of p85 to CD28 and the TCR zeta chain through its E3 ubiquitin ligase activity. The observation that PI3K inhibition reverts enhanced activation of *cblb*-deficient T-cells supports the importance of PI3K as Cbl-b target (see Figure 2) [24]. As a consequence of these molecular interactions, induction of anergy is prevented in T cells deficient in Cbl-b *in vitro* and *in vivo* [22]. As an example for the *in vivo* relevance of Cbl-b in anergy induction, severe collagen-induced arthritis can be induced in *cblb*-deficient mice even in the absence of the adjuvant, again highlighting the hyperactive state of the T cell compartment in these animals [22]. Moreover, in a model of anergy induction in T cells, which makes use of mice carrying the p14 TCRVa2Vb8.2 transgene recognizing the lymphocytic choriomeningitis virus (LCMV) p33 peptide presented by MHC class I, the animals were challenged by repeated injections of the cognate peptide p33. Of note, in this model, *cblb*-deficient p14 transgenic mice challenged with p33 massively activated $CD8^+$ T cells. This induced significant mortality mediated by a severe cytokine storm [22]. When p14/Rip-gp transgenic mice are exposed to LCMV, the animals develop diabetes. The rate of animals developing disease, however, is substantially lower when p14/Rip-gp transgenic mice are challenged with the low agonistic peptide LCMV-LF6 (diabetes rate <50%), whereas injection of *cblb*-deficient animals with this low agonistic peptide induced rapid onset of diabetes paralleled by an enhanced CTL function [25]. From a mechanistic perspective, Cbl-b reduces phosphorylation of PLC-γ1 resulting in reduced PLC-γ1 activity in anergic T cells [22]. Moreover, in addition to an intrinsic hyperactive phenotype, *cblb*

deficiency also induces resistance towards negative cues from the environment, such as soluble TGF-β [26] or effector immune cell suppression mediated by Treg [27]. This is also reflected by decreased generation of inducible Treg in naive *cblb*-deficient T cells, as this process is also mediated by TGF-β [28]. Interestingly, in this particular model the TGF-β pathway was not shown to be defective, but impaired FoxP3 induction was mediated by increased phosphorylation of Foxo3a/Foxo1 in *cblb*-deficient naive $CD4^+$ T cells. In contrast, partial TGF-β resistance has been well documented in $CD4^+$ T cells, which have reduced levels of phosphorylated SMAD2 upon TGF-β stimulation [26]. Thus, Cbl-b clearly interacts with the TGF-β signaling pathway, but the exact molecular background for this observation remains controversial. Nonetheless, resistance of *cblb*-deficient T cells to these negative environmental factors might at least in part explain their increased antitumor efficacy (see below).

4. Cbl-b as Potential Target in Cancer Immunotherapy

Various reports now linked Cbl-b with anticancer immune responses. First, Loeser and colleagues demonstrated that *cblb*-deficient animals are less susceptible to tumor formation in induced as well as spontaneous mouse cancer models relevant for human cancers [29]. Subcutaneous implantation of TC1 tumor cells as well as induction of spontaneous tumors by UV irradiation in *cblb*-deficient animals led to a significantly delayed outgrowth of tumors, when compared to WT animals. Of note, although almost 100% of *cblb*-deficient animals had a delayed tumor growth, only a few animals completely rejected the tumor. It remains to be determined which variables determine complete rejection versus delayed tumor growth. However, tumor rejection was paralleled by an increased infiltration of $CD3^+CD8^+$ T cells into the tumor. Depletion experiments corroborated the functional importance of this particular cell population in tumor rejection, because CD8-depleted animals showed tumor growth comparable to WT animals. In addition, increased tumor infiltration of $CD8^+$ T cells also led to markedly increased amounts of the proinflammatory cytokine IFN-γ within the tumor microenvironment, reflecting the boosted immune response *in vivo*. Finally this led to an impaired tumor cell proliferation as quantified by Ki67 staining and an increased rate of apoptosis, as determined by caspase-3 detection. The same group supported this concept by reproducing these results in mice lacking a functional E3 ligase domain due to a loss-of-function mutation within this region of the *cblb* gene. Again, mice that rejected TC1 tumors had increased $CD8^+$ T cells infiltrating tumors leading to reduced proliferation and increased apoptosis [15]. These data have been further supported by the observation that *cblb*-deficient animals also reject EL4 and EG7 tumor cells [30] as well as spontaneous tumors generated by crossing *cblb*-deficient with ataxia telangiectasia mutant (ATM) mice, which normally develop thymic T-cell lymphoma. Interestingly, these authors also provided genetic evidence that the lack of costimulation is also of *in vivo* relevance, as *cblb*- and CD28-deficient mutant mice are protected against EL4 cells as well. Thus, these mice can generate a productive antitumor immue response even in the absence of CD28 [30]. However, when using an adoptive transfer model, the data are somehow contradictory. First, Chiang and colleagues described that adoptive transfer of 3×10^6 polyclonal *cblb*-deficient $CD8^+$ T cells are hyperactive and reject at least in part EG7 tumors. Moreover, overall survival in this particular model was significantly increased in animals receiving *cblb*-deficient versus WT $CD8^+$ T cells. In contrast, we were not able to detect any protective effect of 5×10^6 polyclonal $CD8^+$ T cells neither in the B16-Ova nor in the EG7 tumor model. We hypothesized that a second *in vivo* activation would be necessary, which allows efficient activation and selection of tumor model antigen positive T cells. Thus, we combined the adoptive T-cell transfer with the application of a dendritic cell (DC) vaccine, which now induced profound antitumor immune effects *in vivo*, which were paralleled by expansion of Ova-specific $CD8^+$ T cells, higher infiltration of $CD8^+$ T cells into the tumor and higher expression of IFN-γ [21]. This observation is also supported by data from Yang and colleagues, who showed that *cblb*-deficient $CD8^+$ T cells are indeed generally more efficacious but are nonetheless unable to mediate curative responses against all tumor types [31]. Using various tumor models expressing an ovalbumin-tagged version of HER-2/*neu* receiving adoptive transfer with WT or *cblb*-deficient $CD8^+$ T cells from OT-I T-cell receptor transgenic donor mice, the authors could demonstrate that at least some tumors (e.g., NOP18) could not be rejected due to insufficient infiltration of Ova-specific T cells into the tumor microenvironment. These data highlight the need of proper migration of tumor-reactive T cells into the tumor microenvironment to attack cancer cells. If this infiltration is prevented, even hyperactive *cblb*-deficient T cells are not able to kill malignant cells.

5. Summary and Perspective

Data from knockout animal studies suggest that inactivation of Cbl-b, which is a nonredundant negative regulator of effector $CD8^+$ and $CD4^+$ T-cell signaling, represents a rational approach to improve anticancer T-cell reactivity *in vivo*. Proof-of-concept studies using adoptive transfer of isolated hyperactive T cells from *cblb*-deficient animals in tumor models validate Cbl-b as potential therapeutic target for improvement of antitumor immunotherapy. Future studies will help to identify strategies allowing *in vivo* pharmacological targeting of Cbl-b activity or genetic modification of Cbl-b expression in adoptively transferred T cells (e.g., by siRNA).

Authors' Contribution

S. Wallner and D. Wolf wrote the first draft of the paper. All authors wrote the final version of the paper.

Conflict of Interests

The authors declare that there is no conflict of interests. There is also no nonauthor involvement in the preparation of this paper.

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