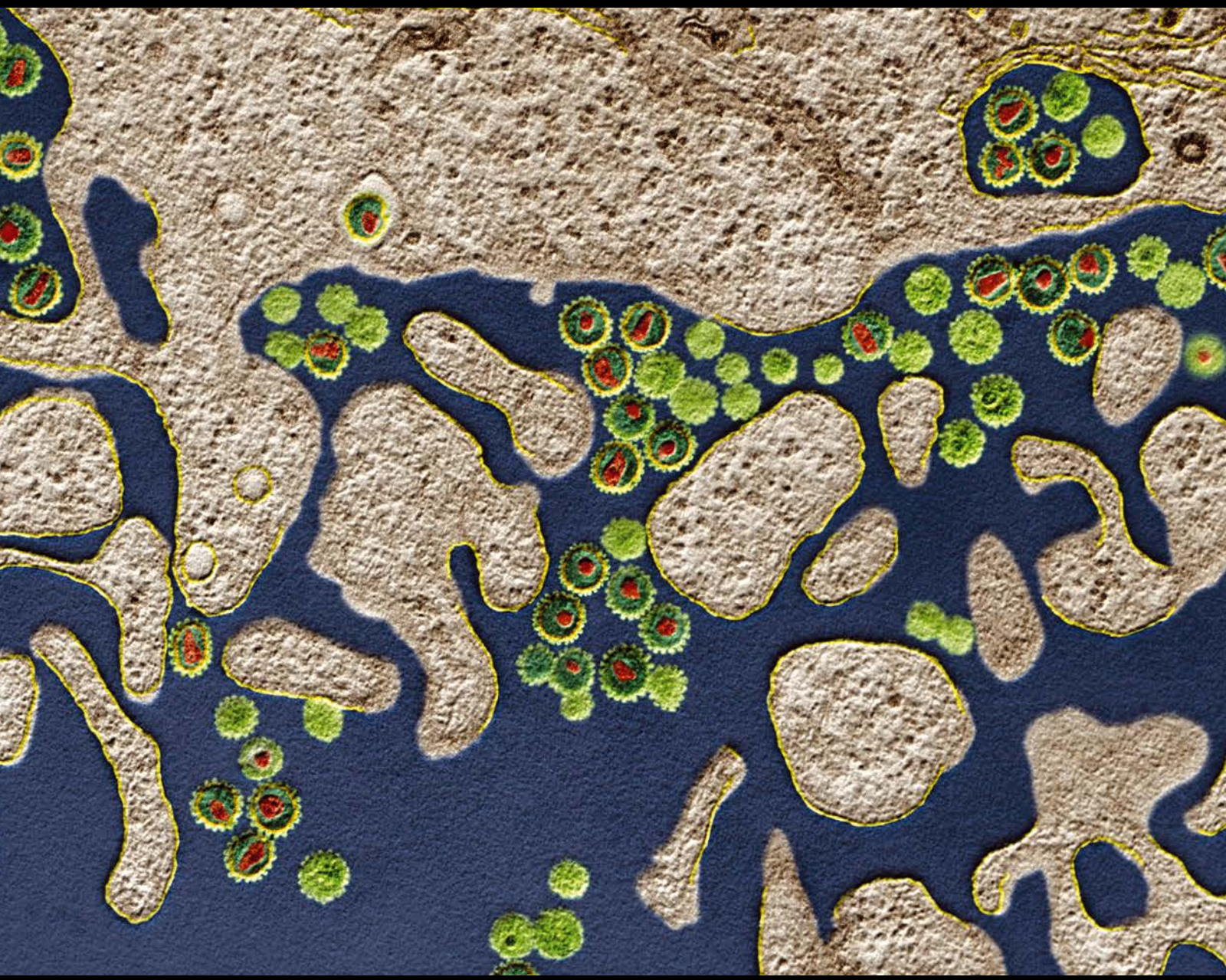
Journal of Immunology Research

Immunotherapy in the Treatment of Human Solid Tumors: Basic and Translational Aspects

Guest Editors: Roberta Castriconi, Barbara Savoldo, Daniel Olive, and Fabio Pastorino



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Editorial Board

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Editorial

Immunotherapy in the Treatment of Human Solid Tumors: Basic and Translational Aspects

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The limited substantial therapeutic progresses observed in the treatment of solid tumors require concerted efforts to combine our thorough knowledge of the tumor biology with a broader comprehension of the main mechanisms regulating antitumor immune responses. In this context, in the last decade, in parallel to the changeless therapeutic quest for targeting molecules responsible for tumor progression, we have witnessed a growing consensus on the potential strong efficacy of combined immunotherapeutic approaches. Recently, these strategies, which include cancer vaccine developments, strenuously pursued for many years, have mainly focused on breaking critical inhibitory immune checkpoint pathways (i.e., PD1/PD-Ls axes), which limit the tumor aggression by different immune cells including natural killer (NK) and T lymphocytes. The strengthening of such endogenous cytolytic effectors, complemented by favoring their migration toward tumors, and/or the infusion of cells engineered with improved function (i.e., with chimeric antigen receptors) is widely considered in additional therapeutic protocols aimed at obtaining a more durable tumor growth control. This goal may also relate to the type of the hematopoietic stem cell transplantation (HSCT) utilized, if included in the treatment of the malignancy.

The papers featured in this issue provide useful information for these rapidly moving above-mentioned fields. Some of them describe new possible mechanisms promoting tumor resistance to effector cell- (like NK cells) mediated aggression or to chemotherapy in the tumor microenvironment favoring tumor progression. Special interest is directed to the possible role for peritumoral NK cells in shaping tumor epithelial mesenchymal transition (EMT) and aggressiveness. C. Goyvaerts and K. Breckpot provide here a useful and comprehensive summary of the current knowledge on the efficacy of vaccination strategies, which differ in the quality of the dispensed antigens and adjuvants, in the modalities of antigen delivery, and in the targeted dendritic cells (DC) subtypes. J. Dannull and colleagues deepen the reasons for the low immunogenicity of immunotherapy strategies based on the infusion of DC-tumor hybrids alone, without adjuvants, whereas another contribution by C. Sakalar and coworkers shows the immunogenic potential of AMHR2 (anti-Müllerian hormone receptor II) molecule in murine models. The efficacy of different vaccination strategies has also been discussed by M. A. Huang and colleagues in the context of pediatric cancer, especially in terms of antibody- and cellular-based therapeutic approaches. These latest interventions include chimeric antigen receptor- (CAR-) T cell based strategies, targeting different antigens in both solid tumors and B cell leukemia. Along this line, B. Clémenceau and colleagues show *in vitro* the efficacy of CAR-NK92 cells

targeting the HER-2 antigen in breast cancer, but discouraging *in vivo* activity in a NOD/SCID/IL-2Rg$^{-/-}$ mouse model, due to an off-target effect of the CH2-CH3 hIgG2 spacer of the CAR construct. By interacting with macrophages at the edge of the tumor, the presence of the spacer in the CAR blocked these engineered cells at the periphery of the engrafted tumors, emphasizing critical issues for therapeutic approaches based on engineered molecules and the need for recruitment of effector cells in the tumor mass to obtain clinical benefits. This last point has been also discussed by C. Cantoni and coworkers who highlight the role of HMGB1 in amplifying the recruitment of NK cells towards tumor, in addition to discussing HMGB1 as a promoter of tumor EMT. HMGB1 also represents the focus of an interesting article provided by V. Pistoia and A. Pezzolo who describe HMGB1 as a factor contributing to tumor resistance in antibody-based immunotherapy. C. Cantoni and coworkers also discuss the complexity of immunosuppressive mechanisms acting in the tumor microenvironment and emphasize a possible role of NK cells in exacerbating tumor aggressiveness from residual cancer cells spared by NK cell mediated killing. This NK-mediated protumoral effect may additionally be caused by the induction/upregulation of ligands (i.e., PD-L1) involved in immune checkpoints. Key immune checkpoints involved in tumorigenesis, and more specifically in glioma genesis, have been clearly discussed by E. S. Kim and colleagues who also provide an overview of the existing preclinical and clinical data, antitumor efficacy, and clinical applications for different checkpoints in glioma. Furthermore, group summarizes results of therapies combining the above-mentioned immunotherapeutic approaches with chemotherapy and radiation. Along this line, a comprehensive review is provided by J. Jacobs and colleagues, specifically focusing their discussion on colorectal cancer.

Overall we have clear indications that the achievement of a long-lasting efficacy in the treatment of cancer patients cannot disregard the best combination of different therapeutic approaches, including HSCT, often planned in the standard of care for different malignancies, such as high-risk neuroblastoma or lymphoma. On this subject, R. Fedele and colleagues provide an exhaustive discussion of the clinical benefit of auto- and allo-HSCT options in relapsed or refractory Hodgkin lymphoma, in parallel exploring results derived from either standard or emerging treatment strategies. Finally, the article provided by R. Ortenberg and colleagues focuses on melanoma and points out the relevance of the timing of therapeutic interventions that may be guided by evaluating appropriate serum tumor biomarkers. In this context, the authors proposed the secreted form of CEACAM1 as a useful tumor biomarker endowed with prognostic role.

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Review Article

NK Cells, Tumor Cell Transition, and Tumor Progression in Solid Malignancies: New Hints for NK-Based Immunotherapy?

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Several evidences suggest that NK cells can patrol the body and eliminate tumors in their initial phases but may hardly control established solid tumors. Multiple factors, including the transition of tumor cells towards a proinvasive/prometastatic phenotype, the immunosuppressive effect of the tumor microenvironment, and the tumor structure complexity, may account for limited NK cell efficacy. Several putative mechanisms of NK cell suppression have been defined in these last years; conversely, the cross talk between NK cells and tumor cells undergoing different transitional phases remains poorly explored. Nevertheless, recent *in vitro* studies and immunohistochemical analyses on tumor biopsies suggest that NK cells could not only kill tumor cells but also influence their evolution. Indeed, NK cells may induce tumor cells to change the expression of HLA-I, PD-L1, or NKG2D-L and modulate their susceptibility to the immune response. Moreover, NK cells may be preferentially located in the borders of tumor masses, where, indeed, tumor cells can undergo Epithelial-to-Mesenchymal Transition (EMT) acquiring prometastatic phenotype. Finally, the recently highlighted role of HMGB1 both in EMT and in amplifying the recruitment of NK cells provides further hints on a possible effect of NK cells on tumor progression and fosters new studies on this issue.

1. Introduction

NK cells are Innate Lymphoid Cells (ILCs) that play a crucial role in the defense against viruses and in the surveillance of tumor insurgence [1–5]. In view of their possible exploitation in cancer (but also in viral infections), these cells have been intensively studied, so that the molecular mechanisms regulating their antitumor cytolytic activity have been extensively defined. By the use of a wide array of surface receptors capable of delivering either triggering or inhibitory signals, NK cells can monitor surrounding cells, checking for their possible phenotypic alterations, and tune an appropriate cytolytic response. In humans, these receptors are essentially represented by the following: (1) the HLA-I-specific inhibitory receptors, Killer Ig-like Receptors (KIR), and CD94:NKG2A receptor, which prevent NK cells from attacking normal autologous cells, and (2) a number of activating receptors (including NKG2D, DNAM-1, and the Natural Cytotoxicity Receptors (NCRs): NKp46, NKp30, and NKp44), which recognize ligands expressed on the surface of transformed and/or virally infected cells and enable NK cells to kill abnormal cells [3, 6].

Most of the above-mentioned receptors are also involved in the control of additional functions exerted by NK cells

ranging from the release of cytokines and chemokines (namely, IFN-γ, TNF-α, GM-CSF, MIP1-α, and RANTES) [3, 7] to the regulatory interactions with different immune cell types including Dendritic Cells (DCs), macrophages, monocytes, granulocytes, and T cells [3, 8–12]. Moreover, NK cells are endowed with additional diverse receptors that enable them to respond to a variegated plethora of stimuli. Thus, NK cells can variably potentiate their functions in response to several Pathogen Associated Molecular Patterns (PAMPs) by using different TLRs (i.e., TLR2, TLR3, TLR7, and TLR9) [13, 14]; they can strongly increase their cytokine production and/or their cytolytic properties in response to different cytokines including IL-2, IL-15, IL-12, IL-18, and IFNs α/β [3, 15]; and finally, they can also migrate in response to various chemotactic stimuli (see below). Two main NK cell functions (i.e., cytotoxicity and IFN-γ production) appear to be differently distributed among specific NK cell subsets in Peripheral Blood (PB) and Lymph Nodes (LN). The so-called "terminally differentiated" PB $CD56^{dim}CD16^{bright}$ NK cells expressing CD57 and KIR molecules display a high cytotoxic potential and a limited ability to secrete IFN-γ upon cytokine stimulation. The $CD56^{dim}CD16^{bright}CD57^{-}KIR^{-}NKG2A^{+}$ PB NK cells exert both functions at intermediate levels. Finally, less differentiated $CD56^{bright}CD16^{dim/neg}CD57^{-}KIR^{-}NKG2A^{++}$ NK cells, which preferentially locate in LN and are poorly represented in PB, show low cytotoxicity and high IFN-γ release upon cytokine stimulation [15–18]. Remarkably, it has been also proposed that NK cells may adapt their cytolytic potential to the pattern of NK receptor ligands (NKR-Ls) stably expressed in the milieu. Thus, the chronic exposure to activating ligands or to abnormally low levels of MHC-I molecules (i.e., inhibitory ligands) would render NK cells poorly reactive. On the other hand, the exposure to adequate MHC-I levels would increase NK cell reactivity and would be essential for differentiated KIR^{+} NK cells to become fully competent [19].

This brief description of the NK cell biology indicates that these cells are far from being simple cytolytic effectors capable of killing different tumor cell targets; rather, they represent a heterogeneous population that is able to fulfill different functions and to finely tune its activity in variable environmental contexts. Such emerging complexity renders the exploitation of NK cells for effective immunotherapies more complicated than initially thought, especially in the context of solid tumors. Indeed, while different animal models and a follow-up study support the notion that NK cells can survey and control the insurgence of tumors [20–22], a straightforward role of NK cells in the control of advanced established solid tumors is far from being defined. In this context the specific tumor associated microenvironment evolving along with the progression of the malignancy may play a role. On the one hand, the increasing tumor structure complexity and the tumor microenvironment can heavily affect NK cell behavior and limit NK cell infiltration of the tumor mass; on the other hand, NK cells that successfully reach (or develop within) the tumor site may interact with different cell populations and influence the progression of the tumor. In the past few years, the suppressive effects of the tumor microenvironment on NK cells have been widely studied [23, 24]. A number of soluble factors [25–28], as well as different tumor associated regulatory/suppressive immune cells [29, 30], Tumor Associated Fibroblasts (TAFs) [31], and tumor cells [32, 33], have been shown to profoundly alter the expression and/or the function of several NK cell receptors and affect the ability of NK cells to reach, recognize, and kill tumor cells at the tumor site (Table 1). Conversely, the possible effect of NK cells on tumor progression is still poorly investigated. In this context, recent evidences indicate that NK cells are capable of modifying the immunogenicity of cancer cells (see below).

The structure of tumor tissue is rather complex and encompasses hypoxic niches, vascularized areas, necrotic zones, and a front of tumor invasion. Along this front, several tumor cells can acquire a less differentiated prometastatic phenotype through a transitional process that in tumors of epithelial origin is referred to as Epithelial-to-Mesenchymal Transition (EMT) (or EMT-like process in melanomas) [48–50]. Thus, the positioning of NK cells within the tumor and their possible conditioning in a hypoxic environment [51] represent important additional elements to be considered in order to define the role of NK cells in the progression of tumor. In this context High Mobility Group Box-1 (HMGB1) [52] may represent a link between NK cells and tumor cell progression. Indeed, recent studies have provided evidence for a role of HMGB1 in the induction of the EMT [53], while in our lab we have recently shown that HMGB1 is actively released during NK:melanoma cell interaction and can amplify recruitment of NK cells.

In this review we analyze the current information suggesting a possible role for infiltrating NK cells in the evolution of tumor cells towards more malignant stages. In this context we also discuss HMGB1 as a possible key-player linking NK cells to the plasticity of tumor cells.

2. NK Cells and the Progression of Cell Tumorigenesis

2.1. The Process of Tumorigenesis and Tumor Cell Plasticity. Normal cell growth and death are tightly controlled processes that ensure the maintenance of tissue homeostasis in the body. The occurrence of random mutations affecting key suppressor genes, oncogenes, and genes involved in DNA repair can alter such homeostatic status, leading to uncontrolled cell growth, immortality, and, finally, tumor onset. In solid tumors the specific microenvironment may enhance the genetic and epigenetic instability of evolving tumor cells, favoring the accumulation of mutations and the progression towards a proinvasive and prometastatic phenotype. Epigenetic changes may contribute to late evolution steps by conferring phenotypic and functional plasticity to tumor cells [48, 54]. The acquisition of plasticity is relevant to the capacity of tumor cells to leave the primary tumor, spread to distant organs through the blood stream or the lymphatic system, and create metastases [55]. In cancers of epithelial origin, tumor cells must lose their epithelial characteristics (polarity, cell-to-cell adhesion, etc.) and gain mesenchymal traits that allow them to detach from the primary site and invade both neighboring and distant tissues. Acquisition of migrating and

Table 1: NK cell infiltrate in solid tumors.

Tumor	NK cell infiltrate: phenotype	NK cell infiltrate: size and/or location	Ref.
Lung adenocarcinoma	Reduced expression of NKp30, NKp46		[34]
	Enrichment of $CD56^{bright}$ $Perf^{low}$ poorly cytotoxic NK cells		[35]
Non-small cell lung cancer	$CD56^{bright}CD16^{dim}$ infiltrating NK cells with impaired killing capability		[36]
	Infiltrating $CD56^{dim}$ with low activating NK-rec expression and function	$NKp46^+$ cells mainly localized at the invasive margin	[37]
Melanoma (primary)		Moderate/low $CD56^+CD3^-$ cells	[32]
Melanoma (primary/metastases)		Low $CD56^+NKG2D^+$ NK cells	[38]
Melanoma (metastases)		Low $CD56^+$ NK cells	[21]
Melanoma (nodal/skin metastases)		$CD56^+$ NK cells rarely present in melanoma	[39]
Melanoma (nodal metastases)	NKp30, NKG2D expression inversely correlated with number of tumor cells in the LN	NK cells surround tumor cell cluster	[40]
Melanoma (nodal metastases)		Enrichment of $CD56^{dim}$ KIR^+CD57^+ cytotoxic NK cells	[41]
Colorectal cancer		Scarce $NKp46^+$ infiltrating NK cells (despite high levels of chemokines)	[42]
Colorectal cancer	Reduced NKp46, NKp30, DNAM-1 expression		[43]
Colorectal cancer (lung metastases)		Low $NKp46^+$ NK cell infiltrate	[44]
Breast cancer	Expression/function of NKp30, NKG2D in infiltrating NK cells decreases with disease progression		[45]
Breast cancer	Enrichment of $CD56^{bright}$ $Perf^{low}$ poorly cytotoxic NK cells		[35]
Renal cell carcinoma (lung metastases)		High $NKp46^+$ NK cell infiltrate correlates with improved survival	[44]
GIST (GastroIntestinal Stromal Tumors)		Substantial $NKp46^+$ NK cell infiltrate mainly surrounding tumor nests	[46]
GIST (GastroIntestinal Stromal Tumors)		Low NK cell infiltration/high metastases at diagnosis	[46]
GIST (GastroIntestinal Stromal Tumors)		High NK cell infiltration/prolonged progression-free survival after imatinib treatment	[47]

invasive properties, as well as turning back into an epithelial phenotype to establish micrometastases, implies a vast cell reprogramming that borrows the molecular pathways from the latent developmental program known as Epithelial-to-Mesenchymal Transition/Mesenchymal-to-Epithelial Transition (EMT/MET) [48].

2.2. The EMT and Its Possible Relationship with the Immune System. The induction of EMT seems to be rather tissue-specific and is governed by complex networks. TGF-β-, Wnt-, Notch-, and growth factor receptor-induced signaling cascades are the main inducers of the EMT; not coincidentally, most of these pathways are overactivated in carcinomas and have been associated with the acquisition of an invasive phenotype [55, 56]. In addition, recent studies have demonstrated that adverse cellular conditions, such as hypoxia or some components of the extracellular matrix (i.e., collagen and hyaluronic acid), can also induce EMT in some cancer types [57–59].

Despite their complexity, the EMT-inducing pathways have a common endpoint in the activation of a short list of EMT-inducing transcription factors (TF) (including Snail, Slug, zinc finger E-box binding homeobox 1 (ZEB1), Twist, Goosecoid, and FOXC2) and MET-inducing microRNAs (miR200 and miR34) that orchestrate the phenotype switch [60]. The consequence of the activation of all these TF is the

transcriptional repression of cellular junction proteins, such as E-cadherin, claudins, and ZO-1, causing the loss of epithelial integrity and also the activation of programs associated with tumor invasion [61–65]. Instead, noncoding microRNAs, microRNA 200 (miR200) and miR205, operate the MET process inhibiting the repressors of E-cadherin expression, ZEB1 and ZEB2, and thereby maintaining the epithelial cell phenotype [66, 67].

Importantly, the recent advances in the characterization of tumor cell plasticity and phenotype switching on epithelial and nonepithelial tumors (i.e., melanomas) suggest that the EMT-MET could be part of a wider, complex transitional phenomenon, in which tumor cells may fluctuate from a differentiated, proliferative, poorly invasive, drug sensitive phenotype to an undifferentiated, poorly proliferative, proinvasive, drug resistant phenotype [68–71]. Thus, tumor cell plasticity and its regulation appear to be a nodal point in the tumor progression and spread; consequently, targeting this phenomenon may be crucial for the immune system to control the fate of the tumor.

Immune cells may have a dual role in carcinogenesis [71, 72]. While a powerful antitumor immune response often occurs to control the first steps of malignant transformation [73–75], in the later tumor stages, transformed cells may manage not only to counteract the immune function but also to use it as an ally in tumor progression [76]. In this context, recent studies have shown that EMT could be locally associated with the presence of granulocytes, Tregs, M2 macrophages, or MDSC, indicating a role for different immune cell types in EMT induction [77–82]. These studies also suggest that EMT may preferentially induce or be induced by suppressive immune cells. Moreover, EMT may directly confer mesenchymal-like immune-modulatory features to tumor cells [83]. Finally, EMT may also modify the immunogenicity of cancer cells, favoring their escape from the T cell-mediated attack (immunoediting) [72, 84, 85]. A link between immunoediting and EMT promotion has been investigated in syngeneic immune-competent mice transplanted with epithelial cancers expressing the neu-oncogene [84]. As expected, the immune surveillance resulted in a macroscopic elimination of the tumor that however was not complete. Indeed, tumor relapse occurred and the new lesions were enriched in neu-negative malignant cells with a mesenchymal phenotype.

Given the potential antitumor and antimetastatic effect of NK cells, an important (still open) question is whether and how NK cells could functionally interact with cells undergoing EMT and how relevant this potential interaction could be to the control of the malignancy.

2.3. NK Cells and EMT. The mutual influence between EMT and NK cells has been barely investigated until now. Only few studies address the issue of the potential changes in tumor immunogenicity during EMT and its implications for NK cell-mediated responses.

In a recent study, López-Soto and colleagues described a significant upregulation of NKG2D activating ligands in colon cancer cells undergoing EMT, as well as a remarkable downregulation of HLA-I expression. They proposed that the EMT could enhance cancer cell immunogenicity towards NK cells and favor tumor clearance in a NKG2D-dependent manner. They also showed that the expression of MICA/B proteins (the main ligands for the NKG2D receptor) was very low in advanced *in vivo* tumors with invasive properties. Concomitantly, a greater presence of $NKG2D^+$ cytotoxic Tumor-Infiltrating Lymphocytes (TILs) was observed in these samples. These results suggested that NK cells, through the engagement of NKG2D receptor, might be responsible for the elimination of MICA/B-expressing transitional cells, thus exerting an immunoediting of tumor cells by the selection of less immunogenic variants [86, 87]. In line with these data, Chen and colleagues showed that prostate cancer cells strongly downregulate HLA-I expression during TGF-β/EGF-induced EMT in a Snail-dependent manner. This phenotypic change might render tumor cells resistant to cytotoxic T cell-mediated lysis but might also increase their susceptibility to NK cell-mediated responses. On the other hand, TGF-β, besides inducing EMT, could also suppress NK cells and compensate the effect of the decreased resistance to NK cells acquired by transitional tumor cells [88].

Compelling evidences indicate that the invasive behavior of tumor cells may be strongly influenced by nearby stromal and immune cells. Nonetheless, regarding the possible effect of NK cells on the induction or inhibition of EMT there are presently no direct data. However, different studies indicated that NK cells can modulate the phenotype of tumor cells and modify their immunogenicity to either NK or T cells. We have recently shown that NK cells can induce HLA-I upregulation on the surface of melanoma cells and confer resistance to NK cell-mediated killing [89]. By coculturing melanoma and NK cells at ratios reflecting the level of NK cell infiltrates observed at the tumor site, we described that the initial tumor cell killing was followed by an equilibrium phase characterized by the upregulation on melanoma cells of both classical and nonclassical HLA-I molecules. This effect was mediated by IFN-γ, which was released by NK cells upon melanoma cell recognition. This NK cell-mediated immunoediting recalls the so-called "adaptive immune resistance," a process in which cancer cells adapt their phenotype under the pressure of the immune response in order to evade it. This phenomenon was hypothesized by Taube and coworkers to describe the acquisition of the inhibitory ligand PD-L1 by tumor cells. In particular, this study showed a clear association between the presence of TILs and IFN-γ close to tumor cells and PD-L1 expression [90]. Thus, the activation of TILs and the consequent release of IFN-γ (but also the expression of IL-10 and IL-32-gamma in the tumor tissue) would result in upregulation of PD-L1 expression [91, 92]. As IFN-γ producers, NK cells might significantly enhance PD-L1 expression on tumor cells. In this context it has been recently shown that supernatants conditioned by IL-2-activated NK cells could increase PD-L1 expression on hematopoietic tumor cell lines and primary Multiple Myeloma (MM), Acute Myeloid Leukemia (AML), and Acute Lymphoblastic Leukemia (ALL) cancer cells [93]. Interestingly, in lung cancer, a recent report showed an important correlation between PD-L1 expression and EMT score [94].

A role for NK cells in the modulation of tumor cell phenotype may be particularly significant in the context of tumor cells that express very low levels of HLA-I and cannot efficiently stimulate T cells. Neuroblastoma cells often show low or negative HLA-I surface expression [95, 96]; in this case, the adaptive immune resistance/immunoediting may be driven by NK cells rather than T cells. However, in spite of the lack of HLA-I expression, neuroblasts isolated from bone marrow aspirates have been shown to be quite resistant to NK cell-mediated killing. This resistance was associated with the lack of ligands for activating receptors (i.e., PVR, recognized by DNAM-1) or with the high expression of B7-H3, a ligand for a still unknown inhibitory NK receptor [96]. Thus, these metastatic neuroblasts may hardly stimulate both T and NK cells, which may result in a lack of IFN-γ production. This situation may account for the recent observation that such aggressive neuroblasts do not constitutively express PD-L1 [97]. Along this line, it has been recently shown that, indeed, metastatic neuroblasts can significantly acquire PD-L1 (and HLA-I) expression in response to IFN-γ [97].

To conclude this issue, it should be considered that an actual evaluation of the possible effects of NK cells on the tumor cell phenotype and plasticity cannot disregard the effective location of the NK infiltrate in the tumor tissue.

3. Infiltration of NK Cells in Solid Tumors

The study of the NK cell infiltrate in solid tumors has been made possible only recently, thanks to the generation of new reagents for the specific detection of NK cells by immunohistochemistry and the availability of even more efficient approaches to isolate and/or analyze specific lymphocyte populations from tissues.

The NK cell infiltrate has been assessed in several types of solid tumors including melanomas [89, 98], GastroIntestinal Stromal Tumors (GIST) [47], and colorectal [99], renal [44], lung [100], and breast cancers [45] (Table 1).

In some cases a role for infiltrating NK cells in the control of tumor progression could be also inferred. Two studies on renal carcinoma lung metastases and GIST found a correlation between the levels of the NK infiltrate and better prognosis [37, 44]. Another interesting study on GIST showed that the number of NKp46$^+$ TILs inversely correlated with the presence of metastases at diagnosis and indicated that different isoforms of the NKp30 activating receptor could associate with reduced or prolonged survival of the patients [46]. Ali and colleagues have also recently shown that tumor-infiltrated LN of melanoma patients are enriched in highly cytotoxic CD56^{dim}CD57$^+$KIR$^+$ NK cells (instead of the poorly cytotoxic CD56^{bright} NK cells which are typically located in LN) [41]. In this context, it should be considered that the cytotoxic activity of the NK cell pool can significantly vary among individuals and that low PB NK cell activity has been associated in the past with an increased risk of many cancers [22, 101].

On the other hand, several data available in the literature call into question the real effect of NK cells on the progression of the tumor. Some studies indicate a scarce or moderate NK cell infiltration in melanomas, colon cancers, and tumor tissues microarray [42, 89, 102]. In addition, studies addressing the phenotype and function of tumor-infiltrating NK cells have shown an enrichment of poorly cytotoxic CD56^{bright} NK cells (in lung and breast tumor tissues) or the presence of altered poorly functional CD56^{dim} NK cells in different tumor types (see Table 1). Finally, independent studies on colorectal cancer, melanoma, and GIST have shown that NK cells may be preferentially located in the stroma, rather than in direct contact with tumor cells [23].

Overall, these conflicting data point out the still open question on how NK cell recruitment and migration are specifically regulated within the tumor tissue. As generally conceived, the migration process would depend on cell-to-cell and cell-to-extracellular matrix (ECM) interactions, modulation of ECM components, the presence of specific chemokines, and the pattern of chemokine receptors expressed by various NK cell types [103].

The chemokine receptor patterns of the most studied PB NK cell subsets (i.e., the CD56^{bright} cells and the composite group of the CD56^{dim} cells) have been roughly defined, although some published studies are not concordant with regard to the expression of certain specific receptors. Such discrepancies may depend on the sensitivity of the antibodies used for flow cytometric analysis or on the use of different cell isolation techniques (which may alter the chemokine receptor recognition by the specific reagent) [104, 105].

Well-established data indicate that the CD56^{dim}CD16$^+$ cells express CXCR1, ChemR23, and CX_3CR1 at high levels and respond to CXCL8 and CX_3CL1 [106]. Accordingly, these cells may cross the endothelium and reach inflamed tissues or tumor masses (as chronic inflammation often characterizes tumor microenvironment). Moreover, CD56^{dim}CD16$^+$ cells also express low levels of CXCR2 and CXCR3 but lack CCR7 and CXCR5. By contrast, CD56^{bright}CD16$^-$ cells express high levels of CCR7 and CXCR3 suggesting that this cell subset would migrate in response to CCL19, CCL21, CXCL9, CXCL10, and CXCL11. CD56^{bright}CD16$^-$ cells also express low levels of CX_3CR1 and lack CXCR1, CXCR2, and CXCR5 [15, 104, 105, 107, 108]. Such chemokine receptor pattern is consistent with the prevalent localization of CD56^{bright} cells in LN, but also with the recent observation that an infiltration of CD56^{bright} cells can be detected in tumors showing high CCL19, CCL21, and CXCL9 transcripts [35].

It should be also considered that NK cells can modify the expression of chemokines and chemokine receptors following cytokine stimulation. For example, short term stimulation of NK cells with IL-2 and/or IL-12 results in a decreased expression of CXCR3 [109], while long term exposure to IL-2 upregulates CCR1, CCR2, CCR4, CCR5, and CCR8 and downregulates the expression of CXCR2 and ChemR23 [110]. In addition, IL-2 can also modulate the expression of CCR7 and induce CCR4 and CX3CR1 expression. IL-15 stimulation causes a decrease of CXCR4 and CX3CR1 expression [111]; IL-18 enhances the response to CCL21 through the induction of CCR7 [112]; TGF-β induces CXCR3 and CXCR4 expression while deeply reducing CX_3CR1 surface levels [25]. Therefore, the composition and the localization of the NK cell infiltrate

would greatly vary, depending on the type of cytokines and chemokines available in the tumor. In mice, using NK cell-sensitive tumor models, it has been shown that IFN-γ can induce the release of CXCL9-10 by tumor-infiltrating immune cells leading to the recruitment of $CXCR3^+$ NK cells [113]. In another study on mice it has been shown that the chemoattractant molecule chemerin can favor the recruitment of NK cells in B16 transplantable melanomas [114]. Interestingly, in humans, the expression of the gene coding for chemerin was downregulated in several tumor types [115]. In a recent study, comparing PB NK cells from healthy donors and neuroblastoma patients it has been shown that patients' $CD56^{dim}$ NK cells display a significantly reduced expression of CX_3CR1, a chemokine receptor involved in the process of cell extravasation [25]. In another study Halama and colleagues have shown a scarce NK cell infiltrate in colorectal cancers despite the high levels of NK cell-attracting chemokines within the tumor. These findings indicate that tumor-orchestrated escape mechanisms may affect NK cell viability in the tumor niche or inhibit the recruitment of NK cells at the tumor site, but also suggest that, besides chemokines, additional chemoattractant molecules may be necessary for the recruitment [42]. Along this line, the role of HMGB1 as chemoattractant for NK cells in tumors has been recently highlighted (see below). Actually, this pleiotropic molecule has recently come into play in various aspects of the tumor biology, not least EMT.

4. HMGB1, Immune Cells, and Tumors

HMGB1 is a widely expressed protein mainly localized in the cell nucleus and involved in chromatin remodeling and transcription [116]. However, following cell activation by various physiopathological stimuli, this protein can undergo posttranslational modifications that promote its translocation to the cytosol and its export outside the cell, via a nonclassic secretion pathway that requires LAMP1 positive lysosomes [117]. HMGB1 can also undergo a passive release from damaged or necrotic cells and behaves as a Damage Associated Molecular Pattern (DAMP) able to trigger and amplify both inflammatory and immune responses [118, 119]. Autophagy induction is required and sufficient to cause the release of HMGB1 from dying cells, suggesting that manipulation of autophagy during cancer treatment may influence the immunogenicity of dying tumor cells [120]. Furthermore, HMGB1 has been identified as a cytokine-releasing factor that stimulates the production of TNF-α, IL-1β, IL-6, and IL-8 from macrophages and neutrophils [121].

The signaling properties of HMGB1 are influenced by the redox state of its cysteine residues localized at positions 23, 45, and 106 [122]. At this respect, all-thiol (i.e., fully reduced) HMGB1 interacts with CXCL12 and the heterocomplex behaves as a chemoattractant for mouse macrophages and fibroblasts and for human monocytes by the engagement of CXCR4 [123–125]. Moreover, HMGB1 can also induce chemotaxis of human monocyte-derived immature DCs through the engagement of RAGE [126]. Conversely, a partial and reversible oxidation of HMGB1 (C23-C45 disulfide bond) is required to activate a TLR4-mediated production of cytokines from macrophages [127]. Instead, HMGB1 released from apoptotic cells is characterized by the irreversible oxidation of C106 to sulfonic acid by ROS. In this oxidation state HMGB1 lacks cytokine-inducing activity, induces tolerogenic DCs, and promotes cell death following treatment with chemotherapeutic agents [128, 129]. Finally, a fully oxidized HMGB1 form has been also described, but its functions have not been yet assessed.

In addition to the redox status, several posttranslational modifications may be relevant to HMGB1 function. So far, the identified modifications on HMGB1 molecules exported from innate immune cells consist of hyperacetylation [130, 131], poly-ADP ribosylation [132], and phosphorylation by CAMK IV and PKC [133–136]. The effects of these molecular changes on the affinity of HMGB1 for its different receptors and their role in cell responses in solid tumors have not been yet explored.

Finally, it should also be considered that HMGB1 can associate with various soluble HMGB1-binding molecules, such as IL-1β, LPS, Pam(3)Csk(4), and the above-mentioned CXCL12, and enhance their immunostimulatory activity [137, 138].

Due to its molecular plasticity, HMGB1, either alone or complexed with other molecules, can interact with several receptors including RAGE, IL-1R, TLR2, TLR4, CXCR4, NMDA-R, and TIM-3 [137–141].

Several different stimuli are able to trigger the release of HMGB1 from cells of the innate immune system. In particular, an active export of HMGB1 is induced on monocytes, macrophages, and DCs activated by PAMPs, DAMPs, or cytokines. In addition, also NK cells can actively release HMGB1 in the context of the NK:DC cross talk or upon the engagement of different activating NK cell receptors, including those mainly involved in tumor cell recognition [130, 142–144]. On the other hand, neutrophils mostly undergo a passive release of HMGB1 following cell injury/necrosis [145], whereas, to our knowledge, no information on the export of HMGB1 from eosinophils and basophils is available. Besides innate immune cells, endothelial cells and fibroblasts can also actively release HMGB1 following the exposure to uric acid and LPS, respectively [146, 147]. It should be also noted that several innate immune cells are targets of HMGB1. In particular, on monocyte-derived immature DCs, HMGB1 upregulates specific maturation markers (CD80, CD83, CD86, and HLA-I), enhances the production of cytokines (IL-6, CXCL8, IL-12 p70, and TNF-α), switches their chemokine responsiveness from CCL5-sensitive to CCL21-sensitive, and induces the cell capacity to stimulate allogeneic T cell proliferation [126]. Moreover, HMGB1 released from primary tumors can reach the regional LN and weaken their antimetastatic capability by lowering the number of resident macrophages [148]. Depending on its concentration, HMGB1 can induce or inhibit neutrophil chemotactic responses by the engagement of RAGE, TLR2, and TLR4 [149]. The essential role of HMGB1 as a crucial modulator of innate immunity in tumors is supported by *in vivo* evidences showing that HMGB1-deficient tumors display an impaired ability to recruit innate immune cells, including macrophages, neutrophils, and NK cells into the tumor tissue following DNA alkylating therapy [150].

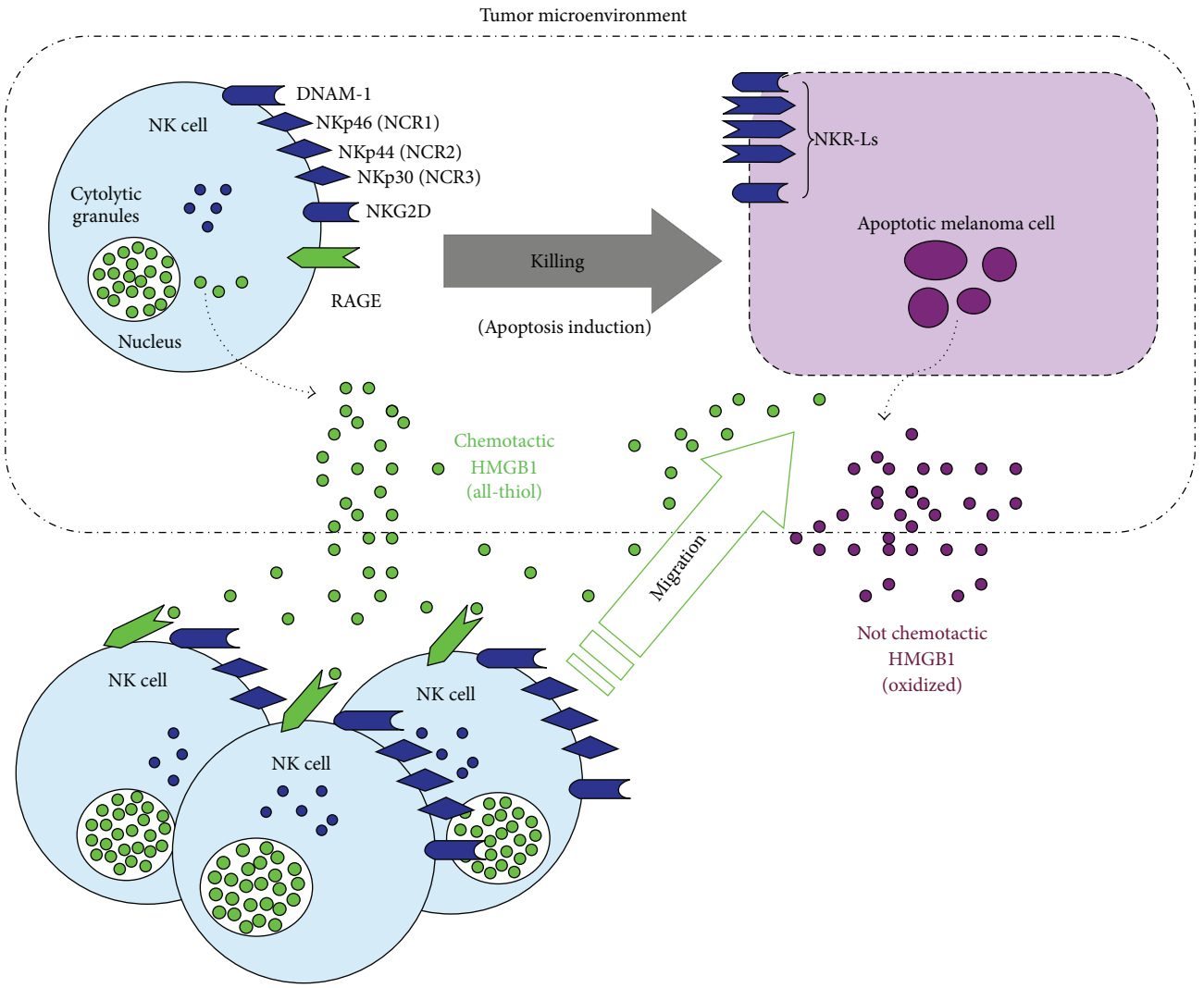

Figure 1: New mechanism proposed for NK cell recruitment at melanoma tumor site. NK cells recognize melanoma cells through the interaction of NK activating receptors with their ligands (NKR-Ls) expressed on tumor cell (both depicted in dark blue). The resulting NK cell activation leads to the killing of melanoma cells (via perforin/granzyme B and induction of apoptosis) and to the active release of a chemotactic form of HMGB1 (green circles). Killed (apoptotic) cells passively release HMGB1 as oxidized molecule. However, this HMGB1 form has no chemotactic properties (purple circles). NK-derived reduced (i.e., all-thiol) HMGB1 can act as chemoattractant for activated NK cells through the engagement of RAGE (depicted in green), thus promoting their recruitment in the tumor microenvironment.

The intracellular amount of HMGB1 is significantly increased in several human tumors such as lung [151], bladder [152], colorectal [153], head and neck [154], prostate [155], hepatocellular [156], and gastric cancer [157] and melanoma [158]. This observation suggests that tumor cells can release high amounts of HMGB1 either by membrane leakage or by active release in inflammatory or hypoxic conditions that are often observed in solid tumors and associated with an increased prometastatic behavior [159]. Extracellular HMGB1 can play further roles in cancer through the activation of endothelial cells. Specifically, the all-thiol form displays a proangiogenic activity that supports tumor growth [160, 161]. The heterogeneous responses observed in different conditions could depend on the local concentration of the different redox forms of the protein, on the type of cellular receptors engaged by HMGB1, and on the presence of specific soluble HMGB1-binding molecules [137, 138]. Thus, HMGB1 from both tumor and immune cells can accumulate in the tumor microenvironment and sustain inflammation, cytokine release, cell proliferation, and recruitment of immune cells. The full characterization of these effects and the evaluation of their importance in the context of the tumor development (or control) may be crucial for the identification of new checkpoints in the host:tumor interaction and for the definition of effective therapeutic targets. In this context we have recently described a new mechanism by which HMGB1 could strongly influence the presence and the efficacy of NK cells at the tumor site.

4.1. Role of HMGB1 in NK Cell-Tumor Interaction. We have recently addressed the role of HMGB1 in the context of the innate immune response against tumors by investigating the function of this protein in the NK:melanoma cell interaction and in the subsequent NK-mediated killing of tumor cells. We have shown that, during the interaction with melanoma cells, NK cells could release an HMGB1 form endowed with chemotactic activity, while killed melanoma cells passively released an oxidized, nonchemotactic form of HMGB1 (Figure 1). The chemotactic HMGB1 could potently attract activated NK cells through the engagement of RAGE, which, indeed, was expressed at the surface of NK cells. Interestingly, after prolonged exposure to HMGB1, NK cells did not enhance their chemotactic properties; rather, they showed an increased cell motility, which was accompanied by expression changes in several proteins involved in the regulation of the cytoskeletal network [144]. Thus, our finding defined a new mechanism by which HMGB1 could sustain the antitumor function of NK cells. Indeed, HMGB1 could initially play a crucial role in amplifying the NK cell recruitment to the site of NK:tumor cell interaction; next, it could improve the patrolling capability of NK cells that have reached the tumor by enhancing their motility.

The presence of HMGB1 in the context of the NK:tumor cell interaction may also play a role in the progression of the tumor. Recent observations show that HMGB1 is a potent driver of EMT in colorectal carcinoma via the activation of the RAGE/Snail/NF-κB pathway and of MMP-7 [53]. NK cells have been shown to be frequently located in the front of invasion of the tumor, where, indeed, the EMT process is likely to occur. Thus, in this situation, HMGB1 may recall additional NK cells in the area, which, in turn, would release further HMGB1 thus contributing to the EMT.

It is worth noting that NK cells also express TIM-3, which has been shown to recognize HMGB1 [162]. Moreover, HMGB1 may also influence NK cell function by its ability to potentiate the activity of the NMDA receptor [141]. Indeed, the activation of this protein channel in human NK cells, T lymphocytes, and neutrophils has been shown to increase the production of ROS [163]. These receptors do not appear to modulate HMGB1-mediated NK chemotaxis, but their possible involvement in additional functions cannot be ruled out.

5. Concluding Remarks

While several reports demonstrate the inefficacy of NK cells in controlling tumor growth and invasion, NK cell role in the prevention of metastasis has been described in different types of cancer, and a higher number of tumor-infiltrating NK cells have been associated with a better prognosis [20–24]. Thus, it is not surprising that in the past years these ILCs have starred in cancer immunotherapy clinical trials with promising results. Therefore, it could be unpopular to ask about their potential role in the tumor progression. Nonetheless, during antitumor immune responses, NK cells can represent a source of IFN-γ, which potentially promotes the adaptive immune resistance of tumor cells, and TNF-α, a known EMT inducer. In addition, NK cells may be often located within the stroma, at the interface with the invasive front of the tumor, where, indeed, the EMT (i.e., the transitional tumor cell phenotype) is frequently observed. Thus several hints foster the idea that NK cells, in spite of their potential ability to control metastases, may also play an unwanted role in the promotion of cancer plasticity. This controversial issue should be definitively clarified. The definition of whether and how NK cells are recruited, migrate within the tumor, and influence the EMT, along with the new insights into the putative role of HMGB1, would provide new important elements to maximize the still unexplored potential of NK cells in the therapy of solid tumors.

Competing Interests

Alessandro Moretta is a founder and shareholder of InnatePharma (Marseille, France) and Daniel Olive is cofounder of Imcheck Therapeutics (Marseille, France). The remaining authors declare no competing interests.

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Review Article

Involvement of HMGB1 in Resistance to Tumor Vessel-Targeted, Monoclonal Antibody-Based Immunotherapy

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High mobility group box 1 (HMGB1) is a member of the "danger associated molecular patterns" (DAMPs) than can localize in various compartments of the cell (from the nucleus to the cell surface) and subserve different functions accordingly. HMGB1 is implicated in maintenance of genomic stability, autophagy, immune regulation, and tumor growth. HMGB1-induced autophagy promotes tumor resistance to chemotherapy, as shown in different models of malignancy, for example, osteosarcoma, leukemia, and gastric cancer. To the best of our knowledge, there is virtually no information on the relationships between HMGB1 and resistance to immunotherapy. A recent study from our group has shed new light on this latter issue. We have demonstrated that targeting of tumor-derived endothelial cells with an anti-human CD31 monoclonal antibody in a human neuroblastoma model was unsuccessful due to a complex chain of events involving the participation of HMGB1. These results are discussed in detail since they provide the first evidence for a role of HMGB1 in resistance of tumor cells to monoclonal antibody-based immunotherapy.

1. Introduction

High mobility group box 1 (HMGB1) is the best characterized member of the so-called "danger associated molecular patterns" (DAMPs), a heterogenous group of molecules that can be derived from any compartment of the cell and are released or secreted by stressed or dead/dying cells in response to sterile inflammation (e.g., trauma, ischemia, autoimmunity, and cancer). DAMPs released in the extracellular milieu alert the immune system of a dangerous situation with the final aim of reestablishing homeostasis. Thus, intracellular DAMPs perform their physiological functions and cannot be detected by the immune system, while extracellular DAMPs act as danger sensors and immunostimulatory molecules [1, 2].

HMGB1 is a highly conserved molecule, present in almost all metazoans and plants. The HMGB1 protein is composed of 215 amino acid residues and is organized in three different domains: (i) A box and B box, two tandem domains, and (ii) a 30-amino acid-long acidic tail in the C-terminal portion of the molecule. HMGB1 box domains bind to DNA in chromatin through protein-protein interactions or recognition of DNA structures. The B box triggers secretion of proinflammatory cytokines by macrophages and this function is competitively blocked by the A box [1, 2]. HMGB1 contains three cysteines: C23 and C45 that can form a disulfide bond and C106 that is unpaired. These cysteine residues are modified by redox reactions that generate three isoforms named "fully reduced HMGB1" for the all-thiol form, "disulfide HMGB1" for the partially oxidized form, and "sulfonyl HMGB1" for the terminally oxidized form [3].

HMGB1 can localize in the nucleus and the cytoplasm and at the cell surface, besides being released extracellularly in a truncated soluble form. Nuclear HMGB1 participates in DNA replication, recombination, transcription, and repair and maintains telomere homeostasis and genomic stability [1, 2, 4]. Under stress conditions, HMGB1 translocates from the nucleus to the cytoplasm where it binds to Beclin-1 (BCN-1) and promotes autophagy (see below), while inhibiting apoptosis [5]. Cell surface HMGB1 promotes neurite outgrowth and platelet activation. Extracellular HMGB1 binds with high affinity to different receptors including the receptor for advanced glycation end products (RAGE), Toll-like receptors

(TLRs) 2, 4, and 9, syndecan-1 (CD138), CD24, and T-cell immunoglobulin mucin-3 (Tim-3) [1, 2, 4]. Notably, CD24 [6] and Tim-3 [7] are negative regulators of HMGB1 effects on macrophages and tumor-associated dendritic cells (DCs). The signal transduction pathways activated by soluble HMGB1 include NF-κB, interferon regulatory factor-3 (IRF-3), and phosphoinositide-3-kinase (PI3K) and culminate into immune cell activation, induction of proinflammatory cytokines and type I IFN, stimulation of cell proliferation, angiogenesis, cell adhesion and migration, and autophagy [1, 2]. Interaction of HMGB1 with RAGE is involved in cell migration, either directly by inducing expression of adhesion molecules such as VCAM-1 and ICAM-1 or indirectly by stimulating secretion of chemokines [1, 2, 4], especially CXCL12 which can form a heterocomplex with HMGB1 endowed with potentiated chemotactic activity [8]. Other partners form heterocomplexes with HMGB1 as interleukin- (IL-) 1, DNA, nucleosome or lipopolysaccharide (LPS), with the eventual result of synergistic proinflammatory and immune activities [1, 2, 4]. The biological functions of HMGB1 vary with the redox states of the cysteine residues; thus, the fully reduced isoform has chemokine activity only, the disulfide isoform shows cytokine activity only, and the all oxidized-isoform is devoid of chemokine or cytokine activity [3].

2. HMGB1 and Cancer

The role of nuclear HMGB1 in DNA repair and maintenance of genomic stability represents by itself a powerful antitumor mechanism [1, 2].

Immunogenic cell death (ICD) is characterized by the release or the exposure on the surface of dying cells of DAMPs that enhance DC differentiation and antigen uptake and presentation, resulting in potent activation of cytotoxic T lymphocyte responses. ICD contributes to tumor eradication in the course of chemotherapy (e.g., anthracyclines, platinum-based compounds) or radiotherapy. Dying tumor cells expose calreticulin on the cell surface and release ATP in the TME; ATP, in turn, binds to the purinergic receptor P2X7 and activates the NLRP3 inflammasome that promotes IL-1β maturation and secretion [9, 10]. Extracellular HMGB1 contributes to chemotherapy-induced ICD by binding to TLR4 on DCs and potentiating antitumor immune responses [11]. However, the overall effects of HMGB1 on tumor growth are complex since, as detailed below, HMGB1 contributes to stimulating neoplastic cell growth and metastasis through different mechanisms, some of which are also involved in the enhancement of antitumor immunity.

The inflammatory tumor microenvironment (TME) induces HMGB1 release by infiltrating leukocytes and the cancer cells themselves. Extracellular HMGB1 in turn binds to RAGE and TLR4 and activates proinflammatory signaling pathways such as NF-κB and inflammasome, thus accelerating tumor growth and metastasis [1, 2, 4, 12]. In pancreatic adenocarcinoma, extracellular HMGB1 has been shown to enhance mitochondrial RAGE expression and translocation, resulting in increased mitochondrial complex I activity and ATP production [13]. These findings, which establish a link between HMGB1 and energy metabolism, are supported by reduced tumorigenesis and ATP production in the TME in RAGE deficient mice [14].

HMGB1 released in the TME induces expression of proangiogenic factors such as vascular endothelial growth factor (VEGF) and their receptors in tumor endothelial cells (EC) through RAGE mediated NF-κB signaling. HMGB1 promotes EC migration and sprouting and sustains a proangiogenic regulatory loop, whereby stimulation of EC with recombinant (r)HMGB1 induces a positive autocrine circuit leading to induction of RAGE and TLR4, as well as of endogenous HMGB1, expression. Therapeutic targeting of HMGB1 inhibits angiogenesis in this model [15].

HMGB1 dampens antitumor immunity by inducing apoptosis of macrophage-derived DCs and suppressing tumor specific $CD8^+$ T cell effectors in part through the induction of IL-10 production by T regulatory cells. Finally, HMGB1 promotes tumor infiltration by lymphotoxin $\alpha 1\beta 2$-producing T cells which recruit M2-type macrophages that support tumor angiogenesis and growth [4].

Autophagy is a programmed process of cell survival based upon lysosome-mediated degradation of cell components (e.g., damaged organelles) and of invading pathogens in a selective or a nonselective modality. Autophagy is primarily controlled by the autophagy-related (ATG) protein family including ATG5, ATG7, and Beclin- (BECN-) 1 but can also occur in an ATG pathway-independent manner [16]. HMGB1 intervenes in the autophagic process at various levels. Nuclear HMGB1 modulates the expression of heat shock protein (HSP) β1 through a pathway that requires phosphorylation of the latter protein at residues Ser15 and Ser86. HSPβ1 is a regulator of the cytoskeleton that controls intracellular trafficking during autophagy and mitophagy, that is, the selective degradation of damaged mitochondria. Inhibition of the HMGB1-HSPβ1 pathway results in deficiency of both autophagy and mitophagy. Cytoplasmic HMGB1 interacts directly with BECN-1 and dissociates it from Bcl-2; the successful accomplishment of this process requires the disulfide HMGB1 isoform. The unc-21-like kinase positively regulates this type of HMGB1-mediated autophagy, whereas TP53 inhibits it. Extracellular reduced HMGB1 induces autophagy and tumor growth through RAGE, whereas oxidized HMGB1 triggers apoptosis of cancer cells [1, 2, 4, 5]. Figure 1 summarizes the main biologic activity of nuclear, cytoplasmic, and secreted HMGB1.

It is now clear that HMGB1 induced autophagy promotes tumor resistance to chemotherapy. This has been shown in different models of malignancy such as osteosarcoma, leukemia, and gastric cancer [17–19]. In contrast, scarce information is available on the relationships between HMGB1 and resistance to immunotherapy. A recent study from our group that is here discussed has shed new light on this latter issue.

3. HMGB1 and Neuroblastoma

Neuroblastoma (NB) is a pediatric malignancy originating from the neural crest that presents with metastatic disease at diagnosis in approximately a half of patients (high risk cases).

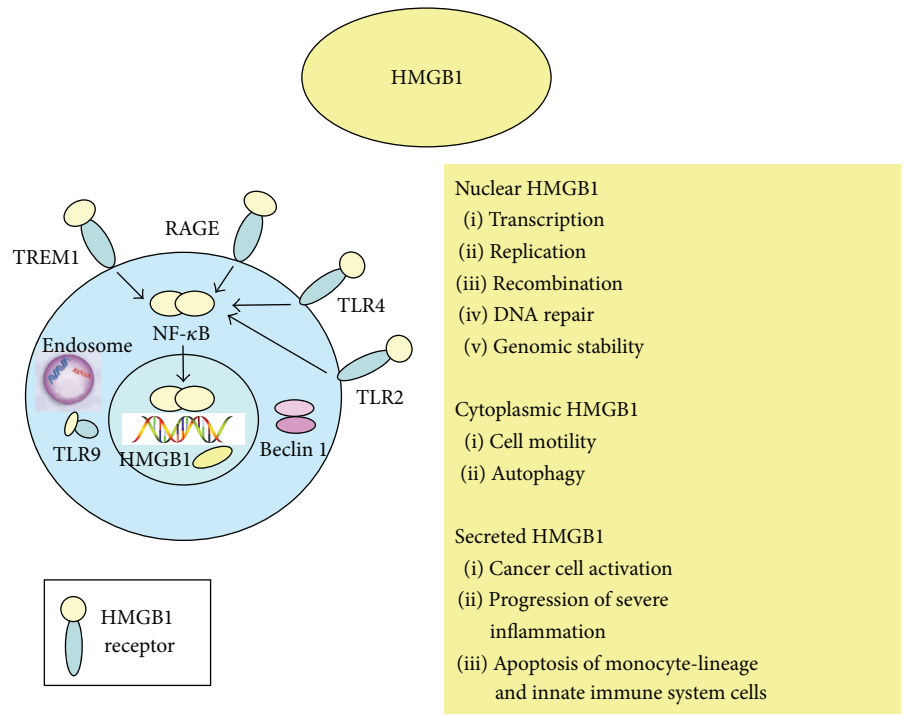

Figure 1: Functions of high mobility group protein B1 (HMGB1). HMGB1 modulates inflammation, immunity, chemotaxis, and tissue regeneration. HMGB1 leads to NF-κB activation that in turn can upregulate inflammatory cytokine production, HMGB1 secretion, and HMGB1 receptor expression (TRL2, TRL24, RAGE, TRL9, and TREM1). HMGB1 participates in DNA replication, recombination, transcription, and repair. HMGB1 interacts with TLR9 in the endoplasmic reticulum-Golgi intermediate compartment. HMGB1 promotes autophagy. Cytosolic HMGB1 binds to Beclin-1 which promotes autophagosome formation and dynamic intracellular trafficking during autophagy.

According to the American Cancer Society (http://www.cancer.org/cancer/neuroblastoma) the 5-year survival of NB patients in the low risk group is higher than 95%, in the intermediate risk group is approximately 90%, and in the high risk group is around 40% to 50%. A therapeutic protocol based upon the combination of anti-GD2 antibody, GM-CSF, IL-2, and isotretinoin has shown great efficacy in a cohort of high risk NB patients, with 2-year event-free survival of 66% and overall survival of 86% [20]. These figures have been recently updated to 74% and 84%, respectively [21].

As in other malignancies, angiogenesis is a major determinant of NB growth and progression. Some years ago, we discovered that variable proportions of NB microvessels were lined by tumor-derived endothelial cells (TDEC), both in tumors formed by the human NB cell line HTLA-230 in immunodeficient mice and in primary tumors (3/10 cases tested). In particular, approximately 50% of the microvessels in experimental tumors and 20–80% in primary NB masses were of tumor origin, as demonstrated by EC expression of classical endothelial markers (CD31, CD105, and von Willebrand factor) and amplification of the MYCN oncogene [22, 23]. Subsequent studies from our group demonstrated that these NB cells disguised as EC and lining microvessels in the TME expressed also typical neuroblastic markers such as GD2, CD56, and NB-84 [24]. Finally, tumor-derived endothelial microvessels were coated by host derived pericytes that never showed MYCN amplification [23].

These results provided an unique opportunity to investigate whether selective targeting of TDEC in our NB orthotopic model closely mimicking primary NB development impacted on tumor growth and mouse survival. TDEC have been implicated in resistance to chemotherapy and tumor progression [25]. Thus, SCID/NOD mice bearing tumors formed by the HTLA-230 cell line were treated with the anti-human (h)CD31 monoclonal antibody (mAb) Moon-1 or with PBS as control [26]. CD31 is a member of the immunoglobulin gene superfamily expressed on the surface of ECs, as well as of various hematopoietic cells including platelets, neutrophils, monocytes, megakaryocytes, natural killer cells, and T and B cells. Numerous heterophilic ligands of CD31 have been identified including the $\alpha v\beta 3$ integrin, the CD38 ectoenzyme, and CD177 expressed on a subset of neutrophils [27, 28]. The *in vivo* relevance of these CD31 heterophilic ligands is unknown. In ECs, CD31 localizes to the borders of adjacent cells and mediates leukocyte migration through ECs and the EC basement membrane. CD31 mediates both outside-in and inside-out signaling. The former are initiated by CD31 ligation and dimerisation and the latter is initiated by integrin ligation, shear stress, cytokines, and other stimuli. The intracellular portion of CD31 contains two immunoreceptor inhibitory motifs that serve as docking sites for signaling molecules such as protein tyrosine phosphatases, whose binding induces phosphorylation of tyrosine- and serine/threonine residues. This latter

event, in turn, promotes recruitment of SH2-containing phosphatases (SHP-1/2 and SHIP) and phospholipase C-γ1, eventually culminating in cell activation [27, 28].

The CD31 mAb Moon-1 does not react with mouse CD31 and therefore targets human EC only, including those derived from NB cells. Survival of mice treated with the Moon-1 mAb or PBS was comparable, indicating the inefficacy of hCD31-targeted immunotherapy. Immunohistological analyses of tumor masses showed that TDEC apoptosis was significantly higher in mAb-treated than control mice, consistent with the cytotoxic activity of Moon-1. Concomitantly, a significant increase of $Ki67^+$, proliferating EC was detected in mAb-treated mice, suggesting the occurrence of vascular remodelling following hCD31 mAb administration whereby TDEC killed by the latter mAb were rapidly replaced by new ones rapidly differentiated from NB cells. In addition, mouse EC were increased after mAb treatment [29].

To gain more insight into the mechanisms underlying such remodelling we first performed gene expression analysis of tumors from treated and control mice, focusing on human angiogenesis-related genes. hCD31 mAb treatment induced outstanding upregulation of the expression of numerous proangiogenic genes including CCL11, CXCL3, CXCL5, cadherin 5, also known as vascular endothelial (VE) cadherin, collagen type IV α3, vascular endothelial growth factor, platelet-derived growth factor-A, fibroblast growth factor-1, tumor necrosis factor, and interleukin-6. In contrast, expression of mouse proangiogenic genes was not modulated by mAb immunotherapy [29]. All of these human proangiogenic factors, that are non-species-specific, likely stimulated both tumor-derived and mouse EC regeneration following TDEC depletion by hCD31 mAb. We also identified a small subpopulation of NB cells that express tenascin-C (TNC) on the cell surface and the stem cell transcription factor Oct-4, which serve as progenitors of TDEC. These cells were found to be increased following hCD31 mAb treatment, consistent with the hypothesis formulated above [29].

TNC is a multifunctional protein of the extracellular matrix that binds to HMGB1 and is involved in epithelial to mesenchymal transition (EMT) [30]. Thus, we investigated the expression of a panel of EMT-related genes in the same tumor samples tested for angiogenesis-related gene expression. EMT-related transcripts, including epidermal growth factor, hepatocyte growth factor, insulin growth factor-1, tumor necrosis factor, CXCL5, IL-6, fibroblast growth factor-1, platelet-derived growth factor-A, and matrix metalloprotease 2, were found to be upregulated in tumors from hCD31 mAb treated *versus* control mice. The next step was to test the expression of few EMT-related proteins, as well as of HMGB1, in tumor tissue sections from hCD31 treated and control mice. It was found that Twist-1, a master regulator of EMT [30], displayed cytoplasmic localization, consistent with a transcriptional inactive state, in tumors from control mice, whereas it was detected in the nucleus of most malignant cells, indicative of ongoing transcriptional activity, in tumors from hCD31 treated mice (Figure 2). The latter tumors lost expression of E-cadherin and acquired expression of N-cadherin, two typical features of EMT, whereas opposite patterns were detected in control tumors (Figure 2). Finally, HMGB1 was always found in cytoplasmic location, but the proportion of HMGB1 cells increased very significantly in tumors from hCD31 mice *versus* controls [29]. Taken together, these studies demonstrated that EMT was involved in the failure of hCD31 mAb treatment and that HMGB1 was an additional player in this phenomenon (Figure 2).

Hypoxia promotes translocation of HMGB1 from the nucleus to the cytoplasm and increases expression of RAGE in the TME [31, 32]. We reasoned that TDEC depletion caused by hCD31 mAb treatment might reduce blood supply and increase tumor hypoxia. Since hypoxia induced factor 2α (HIF2α) is upregulated in NB cells in hypoxic conditions [33] and has been associated with developing endothelium [34], we stained for HIF2α from tumor sections from mice treated with hCD31 mAb or control mice (Figure 1). The proportion of $HIF2\alpha^+$ cells was significantly higher in the former than in the latter tumors, indicating that (i) tumor-derived microvessels were fully functional, (ii) hypoxia was enhanced by selective targeting of TDEC, and (iii) the latter condition was likely involved in HMGB1 upregulation following hCD31 treatment [29]. Hypoxia is also an inducer of EMT [35], so we were interested in investigating whether the results obtained *in vivo* could be replicated *in vitro*. Indeed, human NB cell lines cultured under hypoxic conditions displayed Twist-1 nuclear localization, acquisition of N cadherin, and loss of E cadherin expression (EMT features) compared to the same cells maintained in normoxia. Furthermore, hypoxia induced the appearance of endothelial cell markers (CD31, VE cadherin) and upregulation of cytoplasmic HMGB1 in NB cell lines [29]. Thus, both *in vivo* and *in vitro* data point to hypoxia as a common inducer of EMT and transdifferentiation of NB progenitor cells into TDEC.

HMGB1 induces EMT in lung and renal fibrosis [36, 37], so we asked whether HMGB1 could mimic the effects of hypoxia on human NB cells. To answer this question, the latter cells were cultured with rHMGB1 or medium alone in normoxic conditions and tested for the expression of TNC, as marker of EMT, and VE cadherin and CD31, as markers of TDEC. rHMGB1 induced the expression of all these markers, suggesting that HMGB1 itself was an inducer of EMT and TDEC differentiation in normoxia and recapitulated at least in part the effects of hypoxia on NB cells [29].

A careful review of the recent literature has not disclosed any paper addressing specifically the role of HMGB1 in resistance to immunotherapy. Interestingly, however, it has been shown that cancer-associated fibroblasts that are strongly involved in tumor progression induced HMGB1 upregulation in breast cancer cells, thus contributing to resistance of the latter cells to doxorubicin [38]. Although in our study tumor cells appeared to be the major source of HMGB1, the possibility that stromal cells as cancer-associated fibroblasts contributed to its production cannot be excluded. Another mechanism of tumor resistance to chemotherapy that may apply also to escape from immunotherapy has been recently identified. It has been shown that overexpression of micro(mi)RNA-218 sensitized paclitaxel resistant endometrial carcinoma cells to paclitaxel by binding to the 3′-UTR of the HMGB1 gene, with downregulation of HMGB1 expression and suppression of HMGB1-mediated autophagy [39]. Another recent study

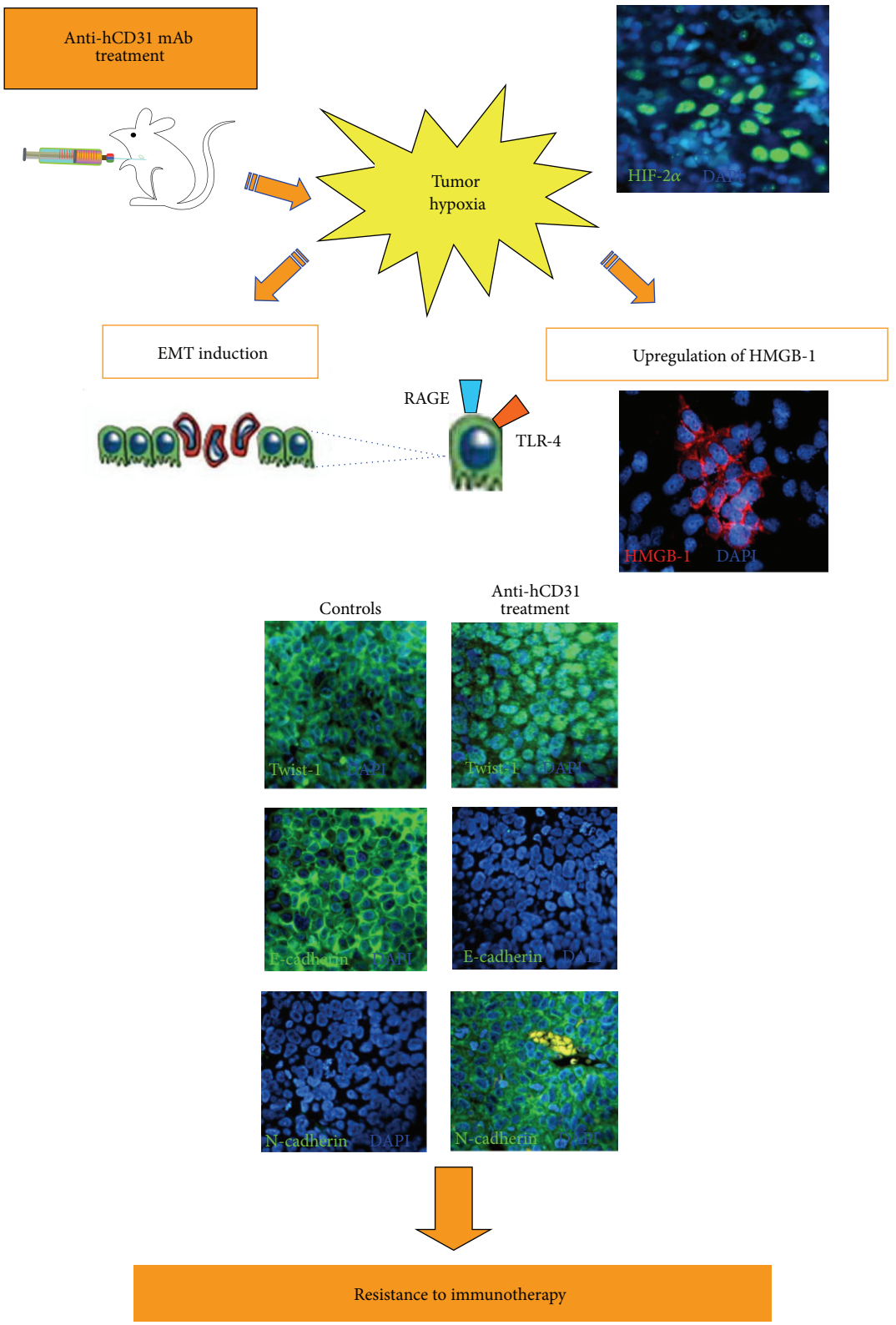

Figure 2: A model for HMGB1 involvement in resistance to mAb-based immunotherapy. hCD31 mAb-driven hypoxia promotes in NB cells expression of HMGB1 that induces by itself EMT, thus mimicking the effects of hypoxia and serving as an amplification loop. All of these mechanisms in combination account for the refractoriness of NB tumors to TDEC targeting with hCD31 mAb.

demonstrated that HMGB1 released from dying cells induced secretory/cytoplasmic clusterin in prostate cancer cells [40]. This latter molecule is a potent antiapoptotic protein that binds to and sequesters Bax from mitochondria, thus preventing caspase 3 activation [41]. HMGB1 induced clusterin protected prostate cancer cells from docetaxel, an antitumor drug [40]. Once again, similar mechanisms might operate in case of resistance of cancer cells to immunotherapy.

4. Conclusions

HMGB1 is involved in resistance to mAb-based immunotherapy in an experimental model where the target antigen is expressed by TDEC. The mechanisms of resistance are numerous and initiated by hypoxia that is a common condition of the TME but was here increased by hCD31 mAb operated depletion of TDEC. Hypoxia, in turn, was the driver of upregulation of EMT-related gene expression, up-regulation of vascular mimicry-related genes (not discussed here), and induction of the expression of cytoplasmic HMGB1, consistent with the chemotactic activity of the latter molecule. All of these events that were closely interconnected converged upon stimulation of differentiation of TNC^+, $Oct4^+$ NB progenitor cells to TDEC, and generation of novel tumor-derived endothelial microvessels, which thwarted the activity of hCD31 mAb (Figure 2). The model proposed to explain the failure of CD31 targeting in our experimental conditions does not take into account the potential involvement of additional players, such as stromal cells or other constituents of the TME.

Unexpectedly, rHMGB1, an equivalent of endogenous extracellular HMGB1, mimicked some of the effects of hypoxia on NB cells, namely, induction of EMT and differentiation to TDEC. We speculated that HMGB1 released in the TME might potentiate the effects of hypoxia in less hypoxic areas of the tumor. Further studies are needed to broaden our knowledge of the role of HMGB1 in resistance to mAb and cellular immunotherapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Immune Checkpoint Modulators: An Emerging Antiglioma Armamentarium

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Immune checkpoints have come to the forefront of cancer therapies as a powerful and promising strategy to stimulate antitumor T cell activity. Results from recent preclinical and clinical studies demonstrate how checkpoint inhibition can be utilized to prevent tumor immune evasion and both local and systemic immune suppression. This review encompasses the key immune checkpoints that have been found to play a role in tumorigenesis and, more specifically, gliomagenesis. The review will provide an overview of the existing preclinical and clinical data, antitumor efficacy, and clinical applications for each checkpoint with respect to GBM, as well as a summary of combination therapies with chemotherapy and radiation.

1. Introduction

Over the past five years, a series of landmark publications heralded the advances of checkpoint inhibitors as cancer immunotherapy [1–3]. Recent clinical trials have demonstrated significant response rates with anti-CTLA-4 and anti-PD-1 antibodies in patients with late stage melanoma and squamous cell lung cancer [1, 4]. These results, along with the recent FDA approval of ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1), continue to highlight checkpoint inhibitors' potential as powerful new additions to the modern anticancer armamentarium.

Preclinical and clinical studies have shown that immunotherapy can improve survival and generate a robust antitumor immune response to improve cancer therapy [5, 6]. Under normal physiologic conditions, immune homeostasis is regulated by a careful balance of activating and inhibitory signals. These "immune checkpoints" (Figure 1) play a critical role in regulating the cells of the immune system. Dysregulation of these checkpoints has been implicated in the pathologically up- or downregulated immune responses seen in chronic infection, autoimmunity, and cancer.

Tumor cells have developed several strategies to exploit these checkpoints and circumvent the host immune defenses. Glioblastoma multiforme (GBM) is the most common central nervous system (CNS) tumor, which has been shown to evade host antitumor response by decreasing immune activation and antigen recognition through several mechanisms. These methods include inducing T cell anergy and lymphopenia, decreasing synthesis of antibodies, increasing immunosuppressive cytokines (i.e., IL10 and TGF-β), upregulating inhibitory molecules of T cells (i.e., Fas ligand [FasL] and programmed death ligand-1 [PDL-1]), and recruiting regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) to subdue immune response [7–13].

The recent discovery of lymphatic vessels in the brain has generated much excitement towards an immune approach to treatment of brain malignancies [14]. This finding provides anatomic evidence for immune communications between the periphery and CNS and may support the long-standing theory that activated, circulating T cells can cross the blood brain barrier after peripheral vaccination or checkpoint inhibition. At present, several studies have demonstrated a positive correlation between high lymphocytic infiltration of primary brain tumors and overall survival [15–21]. Targeted immunotherapy has, therefore, emerged as a promising new approach for treatment, based on the principle that augmenting tumor infiltrating lymphocytes (TILs) activity

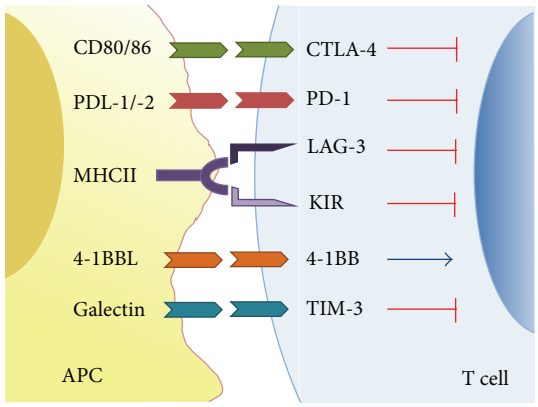

Figure 1: Negative and positive immune checkpoint receptors and ligands.

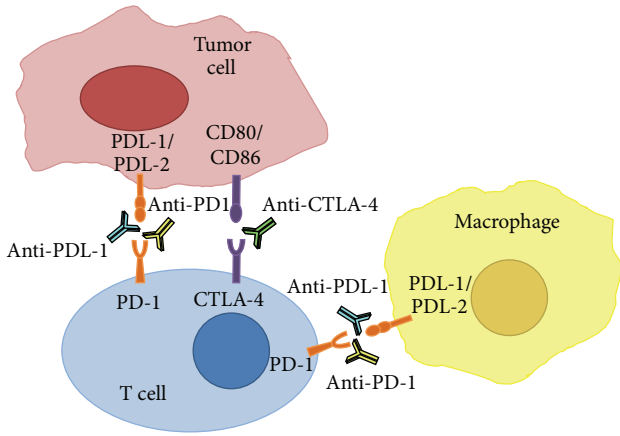

Figure 2: Anti-checkpoint antibodies and their targets.

in the tumor microenvironment could translate to tumor regression [22, 23]. Monoclonal antibodies as agonists or antagonists that target checkpoint inhibitors have emerged as potential strategies to restrict TIL inhibiting signals from the tumor and circulating monocytes, block negative signals and cytokines that inhibit T cell activity, and stimulate systemic immunity (Figure 2) [24].

In this review, we will discuss a series of immune checkpoints that have emerged as potential targets for therapeutic blockade, with an emphasis on those pertinent to the treatment of malignant gliomas. This discussion will touch upon cellular mechanisms, clinical relevance, and outcomes of both preclinical and clinical studies pertaining to each checkpoint. We will also address the topics of combination therapy with other checkpoints molecules as well as other modalities.

2. Immune Checkpoints

2.1. CTLA-4. Cytotoxic T lymphocyte antigen-4 (CTLA-4) is widely regarded as the archetypal T cell intrinsic inhibitory checkpoint. A member of the immune regulatory CD28-B7 immunoglobulin superfamily [25], CTLA-4, acts largely on

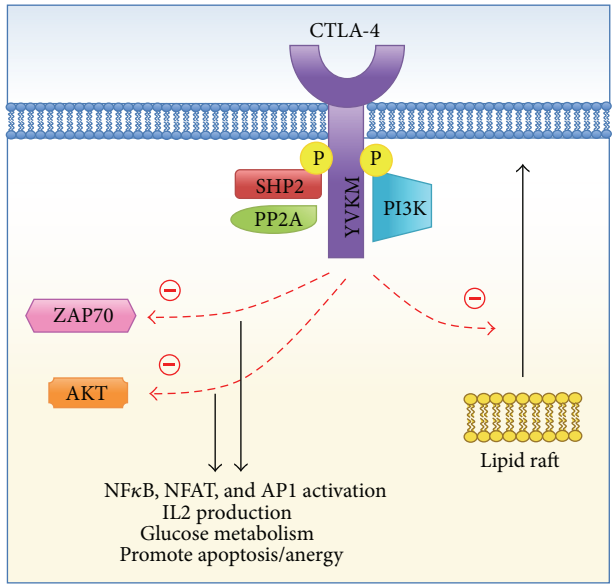

Figure 3: CTLA-4 signaling cascade.

naïve and resting T lymphocytes to promote immunosuppression through both B7-dependent and B7-independent pathways. The B7-1 (CD80) and B7-2 (CD86) proteins found on the surface of antigen presenting cells (APCs) interact with CD28 receptors on T cells to provide the costimulatory "Signal 2" for T cell activation ("Signal 1" being the primary interaction of the T cell receptor [TCR] and Major Histocompatibility Complex [MHC]). Though the B7:CD28 pathway is one of the best-understood mechanisms for T cell costimulation, it is complicated by the addition of CTLA-4 (CD152), a lymphocyte surface protein with 30% homology to CD28 [26]. This transmembrane glycoprotein is a negative T cell regulator that also associates with B7, but with nearly 20 times greater affinity. CTLA-4:B7 engagement not only is quick and effective, but also segregates and prevents B7 from interacting with the activating CD28 [27–29].

B7-dependent immunosuppression occurs through direct engagement of CTLA-4, which may be expressed in a constitutive or rapidly inducible manner on CD4+, CD8+, and regulatory T cells (Tregs) [30]. Though the exact signaling mechanism for T cell inactivation has not yet been fully characterized, existing evidence suggests that upon phosphorylation, CTLA-4 binds to phosphoinositide 3-kinase (PI3K) via a Tyr-Val-Lys-Met (YVKM) motif and activates phosphatases SHP2 and PP2A. Downstream effects of the proposed signaling cascades (see Figure 3) may include inhibition of metabolism [31, 32], inactivation of transcription factors [33, 34], inhibition of CD28-mediated lipid raft formation, [35, 36], and loss of calcium mobilization required for cell proliferation [37].

As an effector molecule, CTLA-4 modulates the threshold for T cell activation [38]. Along with direct signal transduction, engagement with B7 has been shown to control rapid cell surface accumulation of CTLA-4 [39]. CTLA-4 may also actively capture and remove B7-1 (CD80) and B7-2 (CD86) proteins on the opposing APC through a process

of transendocytosis, resulting in "signaling independent" negative T cell regulation [40, 41].

In vivo studies have highlighted the regulatory role that CTLA-4 may play in lymphoproliferation. Early lethality due to uncontrolled polyclonal CD4+ T cell expansion was demonstrated in CTLA-4-deficient mice, ostensibly resulting from dysregulated self-tolerance of peripheral autoantigens [42–44].

2.1.1. Preclinical Evidence. The role of CTLA-4 in glioma maintenance is complex and incompletely understood. While early characterizations of glioma tissue noted dramatic CD4+ lymphopenia and T cell anergy [45–47], the mechanisms by which gliomas achieved global immunocompromise were not yet known. Studies from the early 2000s implicated CTLA-4 in the development of Tregs, a population of immune suppressor cells that is often expanded in gastric [48, 49], pancreatic [50, 51], ovarian [52], and lung cancers [52]. In 2006, using flow cytometry on human GBM samples, El Andaloussi and Lesniak demonstrated that the number of FOXP3+ Tregs were significantly increased in TIL populations compared to controls and that CTLA-4 expression was also elevated within the glioma Treg population compared to those in the control samples [8]. That same year, Fecci et al. reported their findings that while absolute CD4+ cell counts (including CD4+ T helper cells and CD4+CD25+FoxP3+CD4RO+ Tregs) were lower in malignant glioma samples compared to controls, Tregs represented an increased fraction of the existing T cells, and though diminished in number, they were sufficient to significantly impair immune responsiveness [53]. These findings have helped implicate CTLA-4 in the maintenance of an immunosuppressive tumor microenvironment and highlight its potential as a target for immunotherapy in malignant gliomas.

In a follow-up study by Fecci et al, monoclonal anti-CTLA-4 antibody was administered in murine glioma-bearing mice to investigate the immune consequences of CTLA-4 checkpoint blockade. A long-term survival of 80% was reported in the treated group, as well as a restoration of CD4+ proliferation and antitumor capacity. Interestingly, the treatment effects seemed to be exclusive to the CD4+ helper T cell compartment, while Tregs remained functionally unaffected or unsuppressed [9]. Additional animal studies explored the effects of combining anti-CTLA-4 with other immunotherapies. Grauer et al. reported a 50% survival with anti-CTLA-4 alone, compared to 100% survival in mice treated with both anti-CTLA-4 and anti-CD25 (alpha chain of the IL-2 receptor) [54]. Agarwalla et al. found that while high dose anti-CTLA-4 alone was ineffective against large, well-established tumors, the addition of a whole tumor cell vaccination (Gvax) significantly improved long term survival in mice with murine intracranial gliomas [55]. Findings such as these have helped promote the development of clinical trials using anti-CTLA antibody for malignant gliomas.

2.1.2. Clinical Evidence. In light of promising results in animal models, clinical testing of two fully humanized anti-CTLA-4 antibodies, ipilimumab (Bristol Meyer-Squibb) and tremelimumab (Pfizer), began in 2000. The findings from subsequent studies culminated in the 2011 FDA approval of ipilimumab for the treatment of unresectable or metastatic melanoma [3, 6, 41, 56]. With regard to GBM, The National Cancer Institute has begun a Phase I trial to identify safety and dosage of ipilimumab and/or nivolumab with temozolomide in newly diagnosed glioblastoma (NCT02311920). In addition, a randomized, 2-arm, Phase II-III study of ipilimumab in combination with standard-of-care temozolomide for the treatment of newly diagnosed glioblastoma is also currently underway, helmed by the Radiation Therapy Oncology Group (RTOG 1125) [57].

2.2. PD-1/PDL-1. Like CTLA-4, programmed cell death protein 1 (PD-1, also known as CD279) is an inhibitory receptor that negatively regulates the immune system. However, while CTLA-4 mainly affects naïve T cells, PD-1 is more broadly expressed on immune cells and regulates mature T cell activity in peripheral tissues and in the tumor microenvironment [41].

The PD-1 receptor binds two ligands, PD ligand 1 (PDL-1, also known as B7-H1 or CD274) and PDL2 (B7-DC or 273) [58–61], each belonging to the same B7 family as the B7-1 and B7-2 proteins that interact with CD28 and CTLA-4. In the first paper detailing the discovery of the ligand, Dong et al. noted that ligation of PDL-1 not only decreased IFNγ, TNFα, and IL-2 production but also stimulated production of IL10, an anti-inflammatory cytokine associated with decreased T cell reactivity and proliferation as well as antigen-specific T cell anergy [58, 60, 61]. PDL2 ligation also results in T cell suppression, but where PDL-1-PD-1 interactions inhibits proliferation via cell cycle arrest in the G1/G2 phase [62], PDL2-PD-1 engagement has been shown to inhibit TCR-mediated signaling by blocking B7:CD28 signals at low antigen concentrations and reducing cytokine production at high antigen concentrations [59].

Though both CTLA-4 activity and PD-1 activity have immunosuppressive effects, PD-1 relies on different signaling pathways and mechanisms to suppress the T cell inflammatory response and limit autoimmunity (Figure 4).

Ligation of this 288-amino acid transmembrane receptor results in the dephosphorylation (and deactivation) of ZAP70 and the recruitment of SHP2. Upon binding PD-1, SHP-2 directly dephosphorylates PI3K, which inhibits downstream activation of Akt and thereby decreases production of inflammatory cytokine production and cell survival proteins (i.e., Bcl-xL) [63, 64]. Of note, PD-1 activity may be countered or overcome by strong TCR signaling or concomitant CD28 [65] or IL-2 [66] costimulation, allowing recovery of cytokine production and cell survival [61].

2.2.1. Preclinical Evidence. PDL-1 has been shown to be highly expressed on multiple malignant gliomas, as compared to normal brain or benign tumor tissues [67–70]. The mechanism for ligand upregulation has been elucidated in part by Parsa et al., who found that loss of the phosphatase and tensin homolog (PTEN) led to increased PDL-1 gene transcription; furthermore, gliomas with wild-type PTEN were more likely

Table 1: Immune checkpoint antibodies under clinical development.

Target	Biological function	Agent	Stage of clinical development
CTLA-4	Inhibitory receptor	Ipilimumab Tremelimumab	Phase I/II/III/IV Phase I/II/III
PD-1	Inhibitory receptor	Nivolumab (MDX1106, BMS-936558) Pembrolizumab (MK-3475) Pidilizumab (CT-011)	Phase I/II/III/IV Phase I/II/III Phase I/II
PD-L1	Ligand for PD-1	BMS935559 (MDX1105) MPDL3280A MEDI4736 MSB0010718C	Phase I Phase I Phase I Phase I
PD-1-positive T cells	PD-1 inhibitor	AMP-224	Phase I
LAG-3	Inhibitory protein	IMP321	Phase I/II (terminated)
KIR	Inhibitory receptor	Lirilumab (IPH2101, BMS)	Phase I/II
4-1BB	Stimulatory receptor	Urelumab (BMS-663513)	Phase I
GITR	Stimulatory receptor	TRX518	Phase I
TIM-3	Inhibitory receptor	Anti-TIM-3	Preclinical

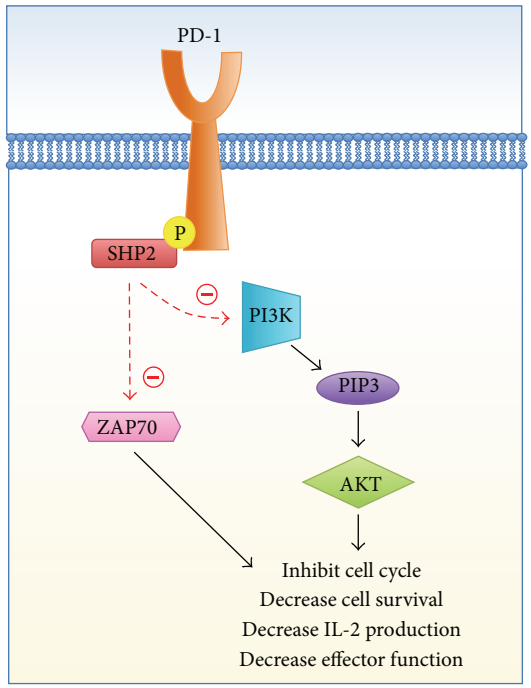

Figure 4: PD-1 signaling cascade.

to be lysed by tumor-specific T cells than gliomas with mutant or inactivated PTEN [68]. The presence of PDL-1 has been associated with potent inhibition of CD4+ and CD8+ T cell activation and cytokine release (IFNγ, IL2, and IL10) [67]. PDL-1 expression levels have also been shown to have significant correlation with tumor grade [71]. Using a mouse orthotopic glioblastoma model, Zeng et al. demonstrated that the combined used of anti-PD-1 and focal radiation therapy led to robust antitumor activity and immunologic memory, as demonstrated by significantly improved survival, increased tumor infiltration of CD8+ T cells, and decreased Tregs populations [5]. These findings have spurred interest in further testing of PD-1 blockade in the clinical trials setting.

2.2.2. Clinical Evidence. At present, several forms of monoclonal anti-PD-1 and anti-PDL-1 antibodies are undergoing clinical development, several of which have shown promising results in early Phase I and II trials (Table 1).

Therapeutic IgGs that target the PD-1 receptor include AMP-224 (Amplimmune), Pembrolizumab (Merck), Nivolumab (BMS), and Pidilizumab (CureTech). Human IgGs targeting the PDL-1 ligand include BMS-936559 (BMS), MEDI4736 (Medimmune), MPDL3280A (Genentech), and MSB0010718C (Merck); additionally, rHigM12B7 (Mayo Foundation) is a human IgM that targets the PDL2 ligand.

Recent results from a clinical trial examining the safety and efficacy of Nivolumab with and without ipilimumab have shown that monotherapy with Nivolumab had fewer treatment related adverse effects than combination therapy and that immune therapy seems to have biologic effects. This has led to Phase III of the trial comparing the safety and efficacy of Nivolumab versus Bevacizumab with or without ipilimumab (NCT02017717). There are several clinical trials recruiting patients to study the effects of anti-PD-1 in patients with GBM. These trials include a Phase I/II clinical trial (NCT01952769) to study the safety and efficacy of Pidilizumab in diffuse intrinsic pontine glioma and relapsed GBM, a Phase II trial of neoadjuvant Nivolumab in primary and recurrent GBM (NCT02550249), a Phase II trial of Pembrolizumab in recurrent GBM (NCT02337686), and several trials examining the effects of combination therapy of anti-PD-1 antibodies with Temozolomide with and without radiation therapy (NCT02311920, NCT02530502), INCB24360 (NCT02327078), FPA008 (NCT02526017), and dendritic cell vaccine (NCT02529072).

3. Additional Checkpoints

3.1. LAG-3. Lymphocyte-activation gene 3 (LAG-3, also known as CD223) is a CD4-related transmembrane protein that competitively binds MHC II and acts as a coinhibitory checkpoint for T cell activation [72, 73]. The mechanism by which LAG-3 negatively regulates the TCR-CD3 complex and inhibits T cell proliferation and cytokine production is not well understood, but several studies have suggested that the inhibitory function depends on a conserved KIEELE motif in the protein's cytoplasmic domain [72–74]. An additional domain binds LAP (LAG-3-associated protein), which may play a role in microtubule association after TCR engagement [75].

LAG-3 is expressed in vivo on the surface of activated CD4+, CD8+, and NK cells [75, 76] under inflammatory conditions. In vitro studies have shown that LAG-3 is upregulated by IL12 and promotes the production of IFNγ [77]. LAG-3 expression is required for maximal Treg function, and ectopic expression may be sufficient for inducing regulatory activity, with suppressive capacities comparable to ectopically expressed FOXP3 [78, 79]. LAG-3 may also play a role in regulating DC function; engagement with DC MHCII molecules has been shown to induce morphologic changes and upregulate IL12 and TNFα secretion [76]. In a study by Workman and Vignali, LAG-3(−/−) T cells exhibited the following characteristics as compared to LAG-3+ cells: (1) delayed cell cycle arrest after stimulation with a superantigen, (2) greater proliferation after in vivo stimulation, (3) and higher numbers of memory T cells after viral exposure [73]. These data suggested LAG-3 plays an important role in regulating T cell expansion, a hypothesis that was further supported by a study by Huang et al. Using LAG-3 knockout mice, the authors demonstrated that, compared to wild-type Tregs, more than double the number of LAG-3(−/−) Tregs were required to control CD4+ helper T cell proliferation at high antigen peptide concentrations; furthermore, the authors reported that administration of anti-LAG-3 antibodies resulted in a reversal of Treg-mediated immune suppression [78]. Grosso et al. also employed antibodies against LAG-3 to increase proliferation and effector function of tumor-specific CD8+ cytotoxic T cells and resulting in disrupted tumor architecture and growth inhibition [80]. A recent study by Woo et al. demonstrated the efficacy of combined checkpoint blockade using three distinct tumor types (B16 melanoma, MC38 colorectal adenocarcinoma, and Sa1N fibrosarcoma); in each of these tumors types, tolerized T cells were found to coexpress LAG-3 and PD-1. Whereas treatment with anti-LAG-3 alone or anti-PD-1 alone delayed tumor growth in a minority of treated mice (0–40%), dual therapy with anti-LAG-3 and anti-PD-1 resulted in complete tumor regression in 70 and 80% of mice with fibrosarcoma and colorectal tumors, respectively. Though no therapeutic effects were observed in the melanoma-inoculated mice, these findings provided compelling evidence for a synergistic benefit of combination checkpoint blockade [81]

3.2. TIM-3. T cell immunoglobulin mucin 3 (TIM-3) was discovered in 2002 as a marker of IFNγ producing CD4+ and CD8+ T cells in mice and humans [82, 83]. A type I glycoprotein receptor that binds to S-type lectin galectin-9 (Gal-9), TIM-3, is a widely expressed ligand on lymphocytes, liver, small intestine, thymus, kidney, spleen, lung, muscle, reticulocytes, and brain tissue [84]. Binding of Gal-9 by the TIM-3 receptor triggers downstream signaling to negatively regulate T cell survival and function. In vitro studies have shown that Gal-9 induced TIM-3 activation induced intracellular calcium influx, aggregation, and cell death (mixed apoptosis and necrosis) of CD4+ T cells; additionally, Gal-9 administration in vivo can cause rapid elimination of IFNγ-producing CD4+ T cells and suppress Th1-mediated autoimmunity [85].

TIM-3 is a marker of CD8+ T cell exhaustion in the setting of chronic viral infections and immunogenic tumor microenvironments [82, 86–90]. TIM-3+PD-1+ TILs have been identified in murine models of colon adenocarcinoma, breast adenocarcinoma, and melanoma; coexpression of these two T cell "exhaustion" markers has been shown to be the most functionally impaired group of CD8+ TIL populations as determined by lowest IL2, TNF and IFNγ production and progression through the cell cycle [90, 91]. In advanced AML tumor models where PD-1+TIM-3+ CD8+ cells have been correlated with disease progression, dual therapy with anti-PDL-1 and TIM-3Ig has been shown to significantly decrease tumor burden and improve survival [86].

Recent evidence suggests that TIM-3 may also play a role in myeloid-derived suppressor cell (MDSC) development. Composed of a heterogeneous group of CD11b+Gr1+ myeloid cells, MDSCs are powerful T cell suppressors that have been shown to proliferate under conditions of infection, autoimmunity, trauma, and malignancy, and their presence has been identified as negative predictive factor predictor for oncologic outcomes [10]. Both Gal-9 and transgenic TIM-3 overexpression have been shown to induce MDSC expansion, with subsequent T cell inhibition [92]; conversely, tumor growth was found to be significantly delayed in TIM-3(−/−) mice implanted with T1 mammary adenocarcinoma, as compared to TIM-3+ wild type mice [92].

3.3. KIR. Killer immunoglobulin-like receptors (KIRs) comprise a diverse repertoire of MHCI binding molecules that negatively regulate NK function to protect cells from NK-mediated cell lysis. KIRs are generally expressed on NK cells but have also been detected on tumor specific CTLs [93]. Members of the KIR family of molecules contain 2-3 Ig ectodomains and cytoplasmic tails of variable length [94]. While some "noninhibitory" KIRs have truncated cytoplasmic tails, others possess longer tails containing two immune receptor tyrosine-based inhibitory motifs (ITIMs) that mediate downstream signaling and confer anti-NK potential [95–98]. The KIR locus is most likely polymorphic and polygenic, with inhibitory KIR haplotypes remaining relatively specific for HLA-B and HLA-C ligands, while noninhibitory phenotypes display greater variability [99].

Unlike adaptive B and T cells, NK cells lack such meticulous antigen sensitivity and instead rely on several activating and inhibitory receptors to modulate and direct their killing capacity [100]. When expressed on the cell surface, KIRs may

play a role in inducing NK tolerance through a process of "licensing," in which each inhibitory receptor recognizes a self HLA class I molecule and prevents NK activation against autoantigens and self-tissue [101, 102]. Knowledge of these germline-encoded receptors has provided valuable insight into the mechanisms of NK-tumor interactions [103, 104]. The phenomenon of NK-dependent rejection of syngeneic or human solid and hematopoietic tumor grafts [105, 106] is partially explained by the "missing" self-recognition phenomenon, where NK cells have been found to target aberrant cells that specifically lack self MHC I expression [107–109]. Though controversial, a few studies have also demonstrated that a lack of KIR ligands or KIR ligand incompatibility with foreign tissues is associated with improved survival and lower relapse rates [110–112] and suggest KIR inhibition as a viable means of enabling or augmenting NK cell-mediated antitumor lytic activity. This hypothesis has been borne out in adoptive transfer experiments of KIR-ligand mismatched or KIR-ligand nonexpressing NK cells which led to significantly increased cytotoxicity of multiple tumor cell lines [113, 114]. KIR blockade using anti-KIR antibodies has also been shown to prevent tolerogenicity and reconstitute NK-mediated cell lysis in both in vivo and in vitro hematopoietic cancer models [115–117].

3.4. 41BB. A member of the tumor necrosis factor (TNF) receptor superfamily that includes the FAS receptor (apoptosis antigen), CD40 (T cell costimulatory receptor), CD27 (TNF receptor), and CD30 (tumor marker), and 4-1BB (CD137) is a Type II transmembrane glycoprotein [118] that is inducibly expressed on primed CD4+ and CD8+ T cells [119], activated NK cells, DCs, and neutrophils [120] and acts as a T cell costimulatory molecule when bound to the 4-1BB ligand (4-1BBL) found on activated macrophages, B cells, and DCs [121, 122]. Ligation of the 4-1BB receptor leads to activation of the NF-κB, c-Jun and p38 signaling pathways [123] and has been shown to promote survival of CD8+ T cells, specifically, by upregulating expression of the antiapoptotic genes BcL-x(L) and Bfl-1 [124]. In this manner, 4-1BB serves to boost or even salvage a suboptimal immune response [120]. Its expression may also be contingent on activation of the B7:CD28 pathway (see above section on *CTLA-4*), with 4-1BB producing its own feedforward loop to maintain T cell activity, and the B7-CD28 complex serving to temper the immune response and protect against inappropriate immune activation [21].

Unlike negative T cell regulators (i.e., CTLA-4, PD-1, LAG-3, and TIM-3), 4-1BB is an activating checkpoint that mediates prosurvival and proinflammatory signaling pathways. 4-1BB costimulation has been shown to profoundly enhance antigen-specific CD8 T cell survival and proliferation [125] and has therefore become a target of interest in tumor immunotherapy, especially against poorly immunogenic tumors for which the host antitumor immune response may prove inadequate. Monoclonal agonist antibodies are one promising method of harnessing the proinflammatory potential of this checkpoint molecule. Anti-4-1BB antibodies have been shown to cause tumor regression in animal models of sarcoma and mastocytoma [119], breast cancer [126], and metastatic colon carcinoma [127] with concomitant increase in tumor selective cytotoxic T cell activity. Synergy with IL-12 gene therapy and anti-4-1BB antibody [127] or local 4-1BB gene [126] delivery has also been shown with significant tumor rejection and long-term immunity seen in metastatic breast and colon cancer models. In intracranial tumor models, anti-41BB has been shown to have moderate cure rates (2/5 mice with GL261 glioma and 4/5 with MCA205 sarcoma), but no effect against the poorly immunogenic B16/D5 melanoma model [128]. Adoptive transfer experiments have also been used to highlight 4-1BB's role in antitumor immunity. CD28 and 4-1BB costimulated T cells adoptively transferred into mice bearing poorly immunogenic melanoma have been shown to result in a 60% cure rate [129] and prolong survival in murine fibrosarcoma models [130]. Whole cell vaccines using tumor cells transfected with 4-1BBL cDNA have also been shown to induce vigorous antitumor CD8+ T cell activity and long term survival in various tumor models [131–134]. However, the technical difficulty and feasibility of culturing and administering lymphocyte or transfected tumor cells for either adoptive transfer or whole cell vaccination have limited their translation into clinical practice.

3.5. GITR. Glucocorticoid-induced TNFR family related gene (GITR) is a member of the tumor necrosis factor receptor (TNFR) superfamily that is constitutively or conditionally expressed on Treg, CD4, and CD8 T cells [135, 136]. Initially described as a unique CD4+CD25+FoxP3+ Treg marker [137], subsequent studies demonstrated rapid upregulation of GITR on effector T cells following TCR ligation and activation [138–142]. The human GITR ligand (GITRL) is constitutively expressed on APCs in secondary lymphoid organs and has also been found on nonlymphoid tissues including vascular endothelial and various epithelial cells [135, 143]. The downstream effect of GITR:GITRL interaction is believed to be at least twofold, including (1) attenuation of Treg activity and (2) enhancement of CD4+ T cell activity [137–139, 141, 144, 145]. The net result is a reversal of Treg-mediated immunosuppression and increased immune stimulation [142, 146].

Like the 4-1BB costimulatory molecule, GITR is an activating checkpoint that enhances inflammatory pathways and host immune response. Overexpression or experimental GITR agonism is associated with autoimmunity [138, 140, 147] and pathologic inflammatory responses such as in asthma [148] and post-stroke states [149]. Preclinical studies have elucidated the differential effects of GITR upregulation on Tregs versus effector T lymphocytes, and its potential role in facilitating the antitumor immune response. Using anti-GITR monoclonal antibodies, Cohen et al. demonstrated that GITR agonism led to lower intratumoral Treg accumulation, loss of FoxP3 expression, decreased Treg suppressor function, and, ultimately, regression of B16 melanoma in mouse models [150]. While these findings were initially implicated Tregs as the primary substrate for GITR:GITRL interactions, subsequent studies have suggested that effector T cells, as opposed to Tregs, may be the principal mediators of the GITR signaling pathway [139–141]. Using GITR knockout mice that still

retained functional Treg populations, Stephens et al. elegantly demonstrated that GITR engagement on CD4+CD25− T cells, and not CD25+ Treg cells, was required to abrogate Treg suppressive activity [151]. Conversely, antagonizing GITRL using blocking antibodies seemed to increase CD4+ T cell susceptibility to Treg-mediated suppression [151]. Additional studies that demonstrated the efficacy of anti-GITR agonist antibodies in inducing tumor regression and preventing regrowth upon secondary challenge have raised interest in GITR as a potential target of tumor immunotherapy [138, 152, 153].

3.5.1. Clinical Evidence. At present, there are no clinical trials for GBM involving IMP321 (a soluble LAG-3 chimeric IgG1 and MHCII agonist), anti-TIM-3 antibody, IPH2101 (anti-KIR), BMS-663513 (a fully humanized anti-4-1BB agonist antibody), or TRX518 (a first in class, humanized anti-GITR monoclonal antibody). However, these immune modulators have tremendous therapeutic potential for the treatment of CNS tumors.

4. Integrating Checkpoint Inhibitors into the Standard of Care

Despite aggressive treatment with chemotherapy and radiation, the refractory nature of high-grade gliomas has become strong motivation to seek novel treatment regimens. The clinical successes of immunomodulating antibodies in both CNS and non-CNS cancers have raised the possibility of adding checkpoint inhibitors to the current anticancer armamentarium as a complementary or even synergistic modality.

Unlike vaccine therapies or adoptive cell transfer, checkpoint inhibition is a nonspecific strategy that relies on generalized activation of the immune system. While T cells are the best-characterized targets of checkpoint inhibition at present, it is becoming clear that these therapies have wide-ranging effects on other immune players such as NK cells, monocytes, macrophages, and dendritic cells [78, 100, 154, 155] (Figure 2). Nonspecific checkpoint-based therapies may therefore benefit from concurrent therapies that either deplete immunosuppressive cells (i.e., chemotherapy) or increase access to tumor-specific antigens (i.e., ionizing radiation).

The following discussion will focus on the possible synergistic effects of concurrent chemoradiation therapy and the challenges of integrating checkpoint inhibitors into the current standard of care.

4.1. Checkpoint Inhibitors and Radiation Therapy. RT is a nonselective cytocidal treatment modality that targets rapidly dividing cells. T cells, which are the main effectors of cancer immunotherapy, are known to be exquisitely sensitive to its effects [156, 157]. Studies testing combined RT and TMZ [158] or RT and steroid [47] regimens have demonstrated significant, long-lasting drops in CD4 counts with concomitant systemic immune compromise. Though these findings could suggest an antagonistic interaction between RT and immunotherapy, the significant cellular and stromal destruction caused by ionizing radiation has been shown to act as a powerful "danger," or activation, signal to the host immune system [159, 160]. Apoptotic tumor cells provide APCs with tumor-specific antigens that can be presented on MHC class I molecules to CD8+ cells, leading to enhanced, antitumor immune activation [161–163]. RT has also been shown to counteract MHC downregulation, a strategy used by GBM to escape immune detection [164, 165]; a study by Newcomb et al. reported a significant upregulation of the β2-microglobulin light chain subunit of the MHCI molecule in GL261 glioma cells following whole body radiation therapy [166].

Elucidating the pathways for radiation-induced immune stimulation provides a mechanism for the observed synergy between radiation and immunotherapy. Prolonged survival with the addition of anti-CTLA-4 to stereotactic radiosurgery has been reported in breast cancer-bearing mice, largely attributed to CD8+ T cell activity [167]. Although it has not been seen in GBM, combination therapy with ipilimumab (anti-CTLA-4 antibody) and local radiation has also been shown to cause tumor regression at both irradiated and nonirradiated sites—the latter known as the abscopal effect [168, 169]. Zeng et al. demonstrated that the addition of SRS to PD-1 blockade increased in vitro expression of proinflammatory molecules such as MHCI, CXCL16, and ICAM and correlated with a survival advantage in glioma-bearing mice [5]. The results of these preclinical studies indicate that RT can work synergistically with checkpoint inhibitors, and at present, a Phase I trial is underway testing the combined used of Pembrolizumab and radiation in GBM (NCT02530502). Results from these studies will help guide future strategies to integrate immunotherapy into the current standard of care therapeutic regimen.

4.2. Checkpoint Inhibitors and Chemotherapy. Approved by the FDA in 2001 for refractory anaplastic astrocytomas and in 2005 for newly diagnosed GBMs, TMZ is a second-generation DNA alkylating agent that is currently the chemotherapeutic standard for the treatment of malignant gliomas. Since its adoption as a first-line agent, population studies have demonstrated an increase in 2-year survival from 7% in cases that were diagnosed between 1993 and 1995 to 17% in those diagnosed between 2005 and 2007 [170]. Use of TMZ in combination with radiation has also been shown to increase two-year survival from 10.4% to 26.5%, as compared to radiation monotherapy [171].

Chemotherapy has been widely hypothesized to be antagonistic or counterproductive to immunotherapy due to its systemic immune toxic effects. Cytotoxic drugs such as TMZ have been associated with severe lymphopenia [172, 173]. In a prospective, multicenter study of patients with high-grade gliomas, Grossman et al. observed long-lasting, systemic CD4+ lymphodepletion with poor clinical outcomes in patients who underwent treatment with oral TMZ and radiation. In this study, median CD4 count was 664 cells/mm^3 before treatment, reached its lowest point at 255 cells/mm^3 two months after the start of TMZ + RT, and remained persistently low for the duration of observation (12 months) [158].

In theory, these effects—in combination with the locally immunosuppressive tumor microenvironment—could abrogate immunotherapy's efficacy by depleting the peripheral pool of effector T cells.

Contrary to these suppositions, numerous clinical studies combining chemotherapy with immunotherapy such as monoclonal antibodies, active specific immunotherapy, and adoptive lymphocyte immunotherapy have shown promising results, though larger studies are needed to verify and assess efficacy [174]. Heimberger et al. published a case study in 2008 demonstrating successful immune activation in a GBM patient following treatment with both TMZ and EGFRvIII vaccine [7]. Of note, the authors observed no significant decline in CD4+ and CD8+ T cell counts and concluded that as long as the cytotoxic chemotherapy was administered outside of the vaccine's therapeutic window, the two modalities could be used in a synergistic manner [7]. Furthermore, some authors have suggested the use of local or intratumoral TMZ as a less immunosuppressive alternative compared to oral TMZ. Using glioma-bearing mice, Brem et al. found that polymeric implants for local TMZ delivery were associated with improved survival, and that the addition of RT prolonged survival even further without additional toxicity [175]. Fritzell et al. later demonstrated that intratumoral TMZ may synergistically increase survival rates in immunized mice by sustained proliferation of CD8+ T cells and decreased intratumoral immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) [176].

With respect to checkpoint inhibitors, these findings imply that carefully timed, interdigitated or alternating chemotherapy would not only protect immunotherapy-activated effector T cells but also ablates immunosuppressive Tregs that could otherwise reduce the efficacy of immunomodulating antibodies [7, 177]. The use of intratumoral chemotherapy may also further protect the effector T cells and provide a survival advantage due to a more robust immune profile. At present, there are no published clinical trials data on the use of TMZ plus checkpoint inhibitors. Further preclinical and clinical studies will be required to examine the risks and benefits of this particular multimodal therapeutic strategy.

5. Summary

Immune checkpoint therapy has emerged as a welcome and potent addition to the current arsenal of anticancer treatment. While certain checkpoint blockades such as CTLA-4 and PD-1 have proven clinically successful, both alone and in conjunction with each other, there are several other targets that such as LAG-3, TIM-3, KIR, and GITR that have shown promise for passive immunotherapy. Anti-CTLA-4 and anti-PD-1 have had promising outcomes in preclinical studies for the treatment of malignant GBMs. Those studies have spurred further ongoing clinical trials that look to solidify immune therapy as a mainstay for treating primary and recurrent brain tumors. Checkpoint inhibitors may be effective not only as monotherapy, but also in combination with chemotherapy and/or radiation therapy. Synergy between the antibodies and either of the two conventional modalities could lead to significant improvements in tumor regression and overall survival. Further research on the mechanisms and therapeutic efficacy of specific antibodies, as well as their interactions with other treatment modalities, is needed to successfully incorporate checkpoint modulators into the current standard of care.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Eileen S. Kim and Jennifer E. Kim contributed equally to this work.

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Review Article

Clinical Options in Relapsed or Refractory Hodgkin Lymphoma: An Updated Review

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Hodgkin lymphoma (HL) is a potentially curable lymphoma, and modern therapy is expected to successfully cure more than 80% of the patients. Second-line salvage high-dose chemotherapy and autologous stem cell transplantation (auto-SCT) have an established role in the management of refractory and relapsed HL, leading to long-lasting responses in approximately 50% of relapsed patients and a minority of refractory patients. Patients progressing after intensive treatments, such as auto-SCT, have a very poor outcome. Allogeneic SCT represents the only strategy with a curative potential for these patients; however, its role is controversial. Based on recent knowledge of HL pathology, biology, and immunology, antibody-drug conjugates targeting CD30, small molecule inhibitors of cell signaling, and antibodies that inhibit immune checkpoints are currently explored. This review will discuss the clinical results regarding auto-SCT and allo-SCT as well as the current role of emerging new treatment strategies.

1. Introduction

Hodgkin lymphoma (HL) is a potentially curable lymphoma with distinct histology, biological behavior, and clinical characteristics. Thomas Hodgkin first described the disorder in 1832. In the 20th century, with the realization that the disease consisted of a lymphoid malignancy, it was renamed HL. It is a relatively rare disease and accounts for approximately 10% of all malignant lymphomas, with about 9,200 estimated new cases and 1,200 estimated deaths per year in the United States [1]. The treatment of HL has evolved over the past three decades, and modern therapy is expected to successfully cure over 80% of patients [2]. Second-line salvage high-dose chemotherapy (HDC) and autologous stem cell transplantation (auto-SCT) have become the standard care for refractory/relapsed HL, leading to long-lasting responses in approximately 50% of relapsed patients and in a minority of refractory patients [3]. Disease recurrence or progression after auto-SCT is associated with very poor prognosis [4] and patients have an estimated average survival of less than 3 years [5]. However, because HL is a rare cancer that is highly curable, the development of new drugs for the treatment of HL has been very slow [6]. With growing knowledge of HL pathology, biology, and immunology, several therapeutic targets have been identified and are currently under preclinical and clinical investigation [7]. The aim of drug development in HL is not only to cure patients, but also to go further and decrease the toxic effects of therapy.

In this review, we summarize the most recent updates on the management of patients with relapsed or refractory HL and the role of novel therapeutic approaches. We also discuss the role of consolidation strategies such as HDC and auto-SCT and reduced-intensity (RIC) allogeneic stem cell transplantation (allo-SCT).

2. Autologous Stem Cell Transplantation

According to retrospective and prospective as well as randomized studies, HDC followed by auto-SCT can rescue 30% to 80% of relapsed/refractory HL patients [8–14].

In the BNLI trial [12], relapsed patients were treated with conventional dose mini-BEAM (carmustine, etoposide, cytarabine, and melphalan) or high-dose BEAM with auto-SCT. Both event-free survival (EFS) and progression-free survival (PFS) showed significant differences in favor of BEAM plus transplant ($p = 0.025$ and $p = 0.005$, resp.). In the GHSG trial [13], patients who relapsed after chemotherapy were randomly given four courses of mini-BEAM+dexamethasone (dexa-mini-BEAM) or two courses of dexa-mini-BEAM followed by BEAM and auto-SCT. Freedom from treatment failure (FFTF) in 3 years was significantly better for patients given BEAM and auto-SCT (55%) than for those on dexa-mini-BEAM (34%; $p = 0.019$). Overall survival (OS) of patients given either treatment did not differ significantly. Recently, the GHSG group [14] evaluated the impact of sequential HDC before myeloablative therapy. Patients with histologically confirmed, relapsed HL were treated with two cycles of dexamethasone, cytarabine, and cisplatin, and those without disease progression were then randomly divided between standard and experimental treatment arms. In the standard arm, patients received myeloablative therapy with BEAM followed by auto-SCT. In the experimental arm, patients received sequential cyclophosphamide, methotrexate, and etoposide in high doses before BEAM. Mortality was similar in both arms (20% and 18%). With a median observation time of 42 months, there was no significant difference in terms of FFTF ($p = 0.56$) and OS ($p = 0.82$) between arms. FFTF in 3 years was 62% and OS was 80%. Results demonstrated that sequential HDC did not improve outcome and was associated with more adverse events and toxicity. Based on the data presented, the authors concluded that two cycles of intensified conventional chemotherapy (DHAP) followed by HDC (BEAM) and auto-SCT are an effective and safe treatment strategy for patients with relapsed HL.

On the basis of this study, BEAM is considered the gold standard conditioning regimen for auto-SCT. However, due to drug constraints of carmustine, this drug is often replaced by a variety of agents, including fotemustine [15], bendamustine [16], and thiotepa [17].

Sweetenham et al. [18] published a retrospective analysis of 175 patients with HL who did not undergo remission after induction therapy and results were reported to the European Group for Bone Marrow Transplantation (EBMT). The 5-year actuarial OS and PFS rates were 36% and 32%, respectively, and results were very similar to those reported from single-institution series and from the Autologous Blood and Marrow Transplant Registry (ABMTR) [19]. The ABMTR series includes 122 patients with HL who have never achieved remission. The definition of failure to achieve remission differs from that in the EBMT series, in that it includes only those patients who had a documented disease progression or tissue confirmation of persistent disease in residual radiographic abnormalities. With a median follow-up of 28 months from the date of auto-SCT, the 3-year actuarial PFS and OS rates in this series were 38% and 50%, respectively. The GELTAMO Cooperative Group [20] presented the results of 62 patients treated with an auto-SCT for refractory HL. One-year transplant-related mortality (TRM) was 14%. The response rate in 3 months after auto-SCT was 52%. Actuarial 5-year time to treatment failure (TTF) and OS were 15% and 26%, respectively. The presence of B symptoms at auto-SCT was the only adverse prognostic factor significantly influencing TTF. The presence of B symptoms at diagnosis, MOPP-like regimens as first-line therapy, bulky disease at auto-SCT, and two or more lines of therapy before auto-SCT adversely influenced OS.

Tandem auto-SCT for HL has been evaluated in a small number of studies [21–24] and in the most recent guidelines from the American Society for Blood and Marrow Transplantation it is not recommended, although further studies may be warranted in high-risk patients [25].

3. Allogeneic Stem Cell Transplantation

Although there is relatively limited accessible data regarding the best approach for patients who relapse after an auto-SCT, the available information supports the benefit of allo-SCT versus standard therapy [25–28]. Evidence of a graft versus HL (GVHL) effect comes from the demonstration that the development of graft versus host disease (GVHD) after allo-SCT is associated with a lower relapse rate [29, 30]. Moreover, the most direct evidence for a graft versus malignancy effect comes from the disease responses to donor lymphocyte infusions (DLIs). Peggs et al. [31] assessed the impact of DLI on relapse incidence when administered for mixed chimerism and the utility of DLI as salvage therapy when given for relapse in 76 consecutive patients with multiple relapsed or refractory HL, who underwent allo-SCT that incorporated in vivo T-cell depletion. The results demonstrated the potential for allogeneic immunotherapy with DLIs both to reduce relapse risk and to induce durable antitumor responses.

Despite early data showing promisingly low relapse rates after allo-SCT, the transplantation community was not very enthusiastic about considering allo-SCT for HL patients, because of the exceedingly high nonrelapse mortality (NRM). Registry data [32, 33] has shown that allo-SCT after myeloablative conditioning results in lower relapse rates but significantly higher toxicity than auto-SCT. Although the poor results after myeloablative conditioning can be explained by the very poor risk features of heavily pretreated patients included in these early trials, high TRM has been associated with high incidence of GVHD and infections after transplantation. Results of allo-SCT can be reasonably optimized with a better patient selection and the use of targeted and less toxic therapies to achieve an adequate response for patients.

In the last years, the use of RIC has reduced NRM and improved OS [34] and the percentage of patients with refractory and relapsed HL treated using this approach has been growing steadily in Europe [35]. Robinson et al. [36] conducted a retrospective analysis of 285 patients with HL who underwent a RIC allo-SCT in order to identify prognostic factors of outcome. Eighty percent of patients had undergone a prior auto-SCT and 25% had refractory disease at transplant. NRM was associated with chemorefractory disease, poor performance status, age > 45, and transplantation before 2002. For patients with no risk factors, the 3-year

NRM rate was 12.5% compared to 46.2% for patients with two or more risk factors. The use of an unrelated donor had no adverse effects on the NRM. The development of chronic GVHD was associated with a lower relapse rate. The disease progression rate in 1 and 5 years was 41% and 58.7%, respectively, and was associated with chemorefractory disease and extent of prior therapy. PFS and OS were both associated with performance status and disease status at transplant. Patients with neither risk factor had a 3-year PFS and OS of 42% and 56%, respectively, compared to 8% and 25% for patients with one or more risk factors. Relapse within 6 months of a prior auto-SCT was associated with a higher relapse rate and a lower PFS.

In the analysis by Robinson et al., the authors also identified important clinical parameters predicting transplant outcomes. RIC allo-SCT may be an effective salvage strategy for the minority of patients with good risk features who relapse after an auto-SCT, with similar outcomes for both sibling and matched unrelated donor (MUD) transplants. On the other hand, for patients with chemorefractory disease or a poor performance status, the overall outcome is poor and it is difficult to recommend RIC allo-SCT for these patients.

Burroughs et al. [37] evaluated the outcome of RIC allo-SCT for patients with relapsed or refractory HL based on different donor cell sources. Ninety patients with HL were treated with nonmyeloablative conditioning followed by allo-SCT from HLA-matched related, unrelated, or HLA-haploidentical related donors. The nonmyeloablative preparative regimen consisted in either 2-Gy total body irradiation (TBI) or combination with fludarabine 30 mg/m2/day followed by postgrafting immunosuppression with mycophenolate mofetil or cyclosporine/tacrolimus. Patients were heavily pretreated with a median of five regimens and most patients had failed auto-SCT and local radiation therapy. With a median follow-up of 25 months, the 2-year OS, the PFS, and incidence of relapsed/progressive disease were 53%, 23%, and 56% (HLA-matched related); 58%, 29%, and 63% (unrelated); and 58%, 51%, and 40% (HLA-haploidentical related), respectively. NRM was significantly lower for HLA-haploidentical related ($p = 0.02$) recipients compared to HLA-matched related recipients. There were promising results with significantly decreased risks of relapse for HLA-haploidentical related recipients compared to HLA-matched related ($p = 0.01$) and unrelated ($p = 0.03$) recipients. The incidence of acute GVHD grade III/IV and extensive chronic GVHD was 16%/50% (HLA-matched related), 8%/63% (unrelated), and 11%/35% (HLA-haploidentical related), respectively.

Raiola et al. [38] confirmed in 26 advanced HL patients the results published by the Baltimore/Seattle group [39], using haplo-mismatched marrow grafts and posttransplantation cyclophosphamide. The procedure was feasible, with a low rate of GVHD and NRM, and was associated with a durable remission in a high proportion of patients. The 4-year OS and EFS were 77% and 63%, respectively. EFS was statistically different when patients were stratified according to disease phase: 1-year PFS was 100%, 67%, and 37% for patients in complete remission (CR) ($n = 9$), partial remission (PR) ($n = 9$), or resistant disease ($n = 8$), respectively ($p = 0.02$). Actuarial survival was not statistically different in the three groups ($p = 0.1$). The cumulative incidence of NRM was 4%. The 100-day cumulative incidence of grade I and grade II–IV acute GVHD was 4% and 24%, respectively; the cumulative 3-year incidence of moderate chronic GVHD was 9%.

The Lymphoma Working Party (LWP) of the EBMT, together with the GEL/TAMO [40], undertook the largest multicenter phase II prospective clinical trial presented up to now with the objective of analyzing the NRM and other major outcome parameters after allo-SCT in relapsed/refractory HL. In this study, 92 patients with an HLA-identical sibling, a MUD, or a one antigen mismatched, unrelated donor were treated with salvage chemotherapy followed by RIC allo-SCT. Fludarabine (150 mg/m2 intravenously) and melphalan (140 mg/m2 intravenously) were used as the conditioning regimen. The addition of antithymocyte globulin was used as GVHD prophylaxis for recipients of grafts from unrelated donors. The NRM rate was 8% in 100 days and 15% in 1 year. Relapse was the major cause of failure. The PFS rate was 48% in 1 year and 24% in 4 years. The OS rate was 71% in 1 year and 43% in 4 years. The results of this study emphasize the role of RIC allo-SCT in patients with relapsed/refractory HL after auto-SCT. The plateau phase in the survival curve of the subset of patients allografted in CR indicates the existence of a clinically beneficial GVHL effect. Chronic GVHD was associated with a significantly lower relapse incidence after transplantation and consequently a significant improvement of PFS.

Recently, the LWP of the EBMT has reported [41] the results on the outcome of the second allo-SCT (allo-SCT-2) performed in one hundred and forty patients with lymphoma, of which 31% were affected by HL. Three-year PFS, OS, relapse incidence, and NRM were 19%, 29%, 58%, and 23%, respectively. PFS and OS were significantly affected by refractory disease at allo-SCT-2 and by a short interval between allo-SCT-1 and allo-SCT-2. Long-term PFS was observed in particular in patients with HL, T-cell lymphoma, and indolent lymphoma where a GVHL effect was assumed [42]. In fact, considering that, in many patients, chronic GVHD was absent after allo-SCT-1 but not after allo-SCT-2, it is possible to conclude that the second allotransplant might induce an effective allo-response in patients in which GVHD failed to appear after the first transplant. Allo-SCT-2 can result in long-term disease control in patients with lymphoma recurrence after allo-SCT-1, in particular if relapse occurs late and is chemosensitive.

4. Brentuximab Vedotin

The expression of CD30 by Reed-Sternberg cells (RSc) coupled with its highly restricted expression makes it an obvious target for monoclonal antibody therapy [43, 44]. Results from two clinical studies using first-generation naked anti-CD30 monoclonal antibodies in patients with relapsed HL have been disappointing, perhaps reflecting their poor antigen binding and/or effector cell activation properties [45, 46]. In an alternate strategy, the anti-CD30 antibody cAC10 was conjugated to a synthetic antimicrotubule agent, monomethyl auristatin E (MMAE), resulting in the novel immunotoxin conjugate brentuximab vedotin [47]. In a

phase I dose escalation trial that enrolled 45 patients with relapsed or refractory CD30+ hematologic malignancies [48], objective responses, including 11 CRs, were observed in 17 patients and tumor regression was observed in 86% of evaluable patients. Seventy-three percent of patients in that trial had undergone auto-SCT. Brentuximab vedotin (1.8 mg/kg intravenously every 3 weeks) was subsequently evaluated in a pivotal phase 2 study of 102 patients with relapsed/refractory CD30+ HL after auto-SCT [49]. Objective responses were documented in 75% of patients, with CRs observed in 34% of patients, as determined by an independent radiology review facility. The estimated 12-month survival rate was 89% and the median PFS was 5.6 months. Adverse events associated with brentuximab vedotin were typically of grade I/II and were treated through standard supportive care. Cumulative peripheral neuropathy, the most meaningful clinical adverse effect, improved or resolved completely in 80% of patients during the study.

Median OS and PFS were estimated in 40.5 months and 9.3 months, respectively. Improved outcomes were observed in patients who achieved a CR on brentuximab vedotin, with estimated 3-year OS and PFS rates of 73% and 58%, respectively, in this group of patients [50]. Of the 34 patients who obtained CR, 16 (47%) remain progression-free after a median of 53.3 months (range, 29.0 to 56.2 months); 12 patients remain progression-free without a consolidative allo-SCT. Younger age, good performance status, and lower disease burden at baseline were characteristic of patients who achieved a CR and were favorable prognostic factors for OS.

On the basis of these studies, brentuximab vedotin has been approved for the treatment of adult patients with relapsed or refractory CD30+ HL following auto-SCT or following at least two prior therapies with auto-SCT or multiagent chemotherapy.

The randomized, double-blind, placebo-controlled, phase 3 AETHERA study [51] demonstrated that brentuximab vedotin improves PFS when given as early consolidation after auto-SCT in patients with HL with risk factors for relapse or progression after transplantation. The high risk of progression after auto-SCT is defined by the presence of primary refractory HL (failure to achieve CR), relapsed HL with an initial remission duration of less than 12 months, or extranodal involvement at the start of pretransplantation salvage chemotherapy. Compared with historical survival data for high-risk patients with HL undergoing auto-SCT, the 3-year OS rate exceeding 80% in this study is remarkable.

A recent SIE, SIES, GITMO position paper declares that there is now evidence for recommending brentuximab vedotin also in HL patients refractory to salvage chemotherapy who are auto-SCT candidates and as a consolidation strategy after auto-SCT. The use of brentuximab vedotin in HL after relapse from allo-SCT or as first-line therapy is at present only experimental [52]. The Expert Panel recommends that, in the approved indications of brentuximab vedotin treatment for HL, treatment evaluation must be performed after 4 courses, and the subsequent treatment should be determined according to the response. In patients with HL attaining a CR, either an early consolidation program including allo-SCT or brentuximab vedotin therapy continued up to 16 cycles is the approved indications. It is necessary to perform clinical trials to clarify which one of the two strategies is more appropriate. Early allo-SCT should strongly be considered in patients with HL attaining a PR. Patients not eligible for transplant should be treated with brentuximab vedotin up to a maximum of 16 cycles. In patients with HL and a stable disease, the decision to continue brentuximab vedotin should rely on a patient-centered balance between clinical benefits and risks. In patients with HL and a progressive disease, brentuximab vedotin therapy should be discontinued and patients must be enrolled in clinical trials.

5. Bendamustine

Bendamustine is a bifunctional alkylating agent with only partial cross-resistance to other alkylating drugs, making it an attractive agent for use in the relapsed setting [53]. Although it was developed in the 1960s and used in Germany for both HL and non-HL, it has been approved for treatment of chronic lymphatic leukemia and indolent B-cell non-HL [54] and limited data exist regarding its activity in HL patients. Moskowitz et al. [55] performed a phase II study evaluating the efficacy and toxicity of bendamustine in relapsed and refractory HL. Thirty-six patients were enrolled, and 25 patients were potentially eligible for allo-SCT. Bendamustine 120 mg/m2 was administered on days 1 and 2 of each 28-day cycle for a total of six cycles of treatment. The dose of bendamustine was reduced to 100 mg/m2 for treatment delays >5 days because of neutropenia or thrombocytopenia. The dose was further reduced to 70 mg/m2 for subsequent delays of >5 days for neutropenia or thrombocytopenia. The most common nonhematologic toxicities were fatigue (primarily grade I) and nausea (primarily grade I). Thrombocytopenia was the most common hematologic toxicity, with 20% of patients experiencing grade III or IV thrombocytopenia. The overall response rate (ORR) for the 36 patients was 53%, demonstrating that bendamustine is a good option for heavily treated patients with relapsed and refractory HL who could proceed to consolidative SCT. Zinzani et al. [56] reported two cases of patients relapsed/refractory after brentuximab vedotin were successfully treated with bendamustine indicating that patients with HL relapsed/refractory to brentuximab vedotin therapy may be chemosensitive and may obtain a good response to subsequent bendamustine treatment. Zinzani et al. [57], after these case reports, performed a retrospective study on 27 heavily pretreated patients with relapsed or refractory HL, who had all received brentuximab vedotin as their last treatment and who showed disease progression, refractory disease, or early relapse, when retreated with bendamustine. The ORR was 55.5%, with 10 of 27 patients (37.0%) obtaining a CR. In comparison, the ORR previously observed with brentuximab vedotin in the same subset of patients was much lower (18.5%).

Considering the promising results of brentuximab vedotin and bendamustine as single drugs on patients with relapsed/refractory HL and their independent mechanisms of action with manageable safety profiles, a phase I-II study was performed evaluating the safety and efficacy of brentuximab vedotin in combination with bendamustine

for the treatment of patients with HL first relapse [58]. Brentuximab vedotin 1.8 mg/kg on day 1 in combination with bendamustine 90 mg/m2 on days 1 and 2 of 3-week-cycles for up to 6 cycles had a manageable safety profile with premedication. The CR rate of the combination was 82% and ORR 94%. The majority of CRs (24/28 patients) were documented after 2 cycles of combination therapy and, in these patients, stem cell mobilization and collection were performed with success. These data indicate a promising approach for maximizing responses prior SCT in relapsed/refractory HL patients after frontline therapy. Promising data were reported by O'Connor and colleagues [59] on the combination of brentuximab vedotin and bendamustine in relapsed/refractory HL and anaplastic large T-cell lymphoma.

6. Panobinostat and Mocetinostat

Agents that target acetylases may regulate several oncogenic pathways including cell cycle progression, cell survival, angiogenesis, and antitumor immunity. Panobinostat and mocetinostat target histone deacetylase (HDAC) and these agents may be effective in patients with HL by modulating serum cytokine levels and the expression of PD-1 on intratumoral T-cells.

Based on promising results from a phase I study that included 13 patients with relapsed HL [60], a large pivotal international phase II study was initiated. Oral panobinostat was administered at a dose of 40 mg three times per week, every week, in 21-day cycles. Dose delays and modifications for management of adverse events were permitted, but the lowest dose allowed on study was 20 mg. Efficacy was evaluated every 2 cycles by imaging studies. Surprisingly, patients were enrolled in less than one year. The median age was 32 years (range, 18–75), and the median number of prior chemotherapeutic regimens was 4 (range, 1–7). Importantly, the median time to relapse after the first auto-SCT was only 8 months, which represents a poor prognostic indicator. Moreover, 37% of the patients did not respond to their last prior therapy. Twelve patients also received prior allo-SCT.

In a phase II study, 129 patients with relapsed and refractory HL received 40 mg of panobinostat orally three times per week [61]. Treatment with panobinostat was effective as tumor reductions were seen in 74% of patients, and ORs were achieved by 35 patients (27%). Thirty patients (23%) had partial responses to treatment and five patients (4%) had CRs. The median duration of response was 6.9 months, and the median PFS was 6.1 months. The treatment was reasonably well tolerated with common drug-related grade I/II adverse effects as diarrhea, nausea, fatigue, vomiting, and anorexia. Common drug-related grade III/IV adverse events were thrombocytopenia, anemia, and neutropenia. The thrombocytopenia was manageable and reversible with dose hold and modification.

Considering the synergistic activity of HDAC inhibitors with other therapies [62, 63], association studies of HDAC inhibitors combined with chemotherapy, monoclonal antibodies, and small molecule inhibitors will be evaluated. A phase I study of panobinostat combined with lenalidomide in relapsed HL is ongoing [64].

The safety and efficacy of mocetinostat were recently evaluated in a phase II study in 51 patients with relapsed classical HL [65]. Mocetinostat was given orally 3 times per week (85 mg to 110 mg starting doses) for 1 year in the absence of disease progression or prohibitive toxicity. Initially, 23 patients were enrolled in the 110 mg cohort. Subsequently, because toxicity-related dose reductions were necessary in the 110 mg cohort, 28 additional patients were treated with a dose of 85 mg. The disease control rate was 35% (eight of 23 patients) in the 110 mg group and 25% (seven of 28) in the 85 mg group. Three of the 10 (30%) patients in the 85 mg group achieved partial remissions. Furthermore, grade III and IV toxicity (mainly fatigue, with no significant hematologic toxicity) was reduced to 20%. Overall, 80% of the 30 evaluable patients had some decrease in their tumor sizes. These data demonstrate that mocetinostat has a promising single-agent clinical activity with manageable toxicity in patients with relapsed classical HL.

7. Everolimus

The phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) signaling pathway is one of the most aberrantly activated survival pathways in cancer, making it an important target for drug development [66]. Everolimus is an oral antineoplastic agent that targets this pathway, specifically the mTORcomplex1 (mTORC1) that has been shown to be activated in patients with HL. Everolimus not only may target the signaling pathways within the RSc but may also suppress signaling within the immune infiltrate and production of cytokines present in the tumor microenvironment [67]. Nineteen evaluable patients with relapsed HL were treated with daily doses of 10 mg everolimus, the ORR rate was 47%, and 8 patients achieved PR and 1 CR [68]. The median time to disease progression was 7.2 months. The majority of patients had received multiple previous lines of therapy and 84% of the patients had undergone a previous auto-SCT. Grade III adverse events included thrombocytopenia and anemia. Considering that several signal transduction pathways are critical for the proliferation and survival of neoplastic Hodgkin RSc, including NF-κB, JAK-STAT, PI3K-AkT, and ERK [69], a combination of therapeutic approaches capable of targeting RSc along with reactive cells of the microenvironment might prolong the response duration of mTOR inhibitors to overcome chemorefractoriness.

8. JAK Inhibitors

The Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway is an active mediator of cytokine signaling in the pathogenesis of solid and hematologic malignancies. The seven-member STAT family is composed of latent cytoplasmic transcription factors that are activated by phosphorylation intertwined in a network with activation that ultimately leads to cell proliferation. Aberrant activation of the JAK-STAT pathway has been demonstrated in patients with large granular lymphocytic leukemia, aplastic anemia, myelodysplastic syndrome, myeloproliferative disorders, and HL [70]. Pacritinib is an inhibitor of JAK2

kinase with preclinical activity in a variety of hematological malignancies [71]. This JAK inhibitor has been used in a phase I clinical trial in patients with relapsed or refractory Hodgkin or non-Hodgkin lymphoma of any type except Burkitt or central nervous system lymphoma [72]. Doses of 100 to 600 mg/day were tested, and treatment was well tolerated, with mostly grade I/II toxicities. Among the 34 patients' study, the ORR was 14%, including three partial remissions. In the group of patients with HL, however, none of the 14 patients had a partial remission or better. However, at least five of the patients with HL did benefit from the treatment, with a decrease in the sites of active disease.

9. Rituximab

Rituximab has shown activity in nodular lymphocyte predominant HL. It is active in relapsed/refractory classical HL regardless of subtype or degree of CD20 expression on RS cells. Rationale of using rituximab in classic HL includes elimination of CD20+ reactive B-cells supporting RS cells, hence depriving malignant cells of survival signals and potentially increasing host immune responses [73]. In a pilot study [74], 22 patients with recurrent, classic HL who had received a minimum of two prior treatment regimens, regardless of whether H/RS cells expressed CD20, were treated with 6 weekly doses of 375 mg/m2 rituximab to selectively deplete infiltrating benign B-cells. Five patients (22%) achieved partial or complete remission that lasted for a median of 7.8 months (range, 3.3–14.9 months). Remissions were observed in patients only at lymph node and splenic sites, but not at extranodal sites, and were irrespective of CD20 expression by H/RS cells. Furthermore, systemic (B) symptoms resolved in six of seven patients after therapy. These data need to be confirmed in clinical trial.

10. Lenalidomide

Lenalidomide is an immunomodulatory agent with several mechanisms of action, including direct induction of apoptosis in tumor cells, antiangiogenic effects, and the modulation of immune cells, such as natural killer cells and T-cells [75]. Limited data suggest that lenalidomide has clinical activity in relapsed/refractory HL. Fehniger et al. [76] evaluated 38 relapsed HL patients with 25 mg/day of lenalidomide on days 1–21 of 28-day cycles; 33 of 38 patients had prior SCTs. The ORR to lenalidomide in the 35 evaluable patients was 17%, with one CR. Additional six patients had stable disease (SD) lasting >6 months, resulting in an overall cytostatic response rate (CR + PR + SD > 6 months) of 34%. Treatment continued until progressive disease or an unacceptable adverse event. Kuruvilla et al. [77] evaluated lenalidomide in 14 patients with relapsed or refractory HL. Two patients achieved a PR (14%), with additional seven patients having SD (50%). The median time to progression in that study was only 3.2 months, with a median OS time of 9.1 months. Böll and colleagues used lenalidomide in 42 patients [78]. Preliminary results involving the first 24 patients have been reported. Twelve patients (50%) had an objective response (11 with a PR and one with a CR), with additional eight patients achieving SD. Further studies must be made to evaluate the actual efficacy and long-lasting effect of lenalidomide.

Lenalidomide was further evaluated in HL by the GHSG in a first-line phase I combination trial for older patients [79]. The GHSG aimed to improve the ABVD regimen by replacing bleomycin with lenalidomide (AVD-Rev) to improve both efficacy and tolerability of the regimen. Patients received four to eight cycles of AVD-Rev (standard-dose AVD on days 1 and 15 of a 28-day cycle and lenalidomide daily from days 1 to 21) followed by radiotherapy. The daily lenalidomide dose for the first patient was 5 mg; maximum dose in this dose escalation trial was 25 mg. Twenty-five patients with a median age of 67 were enrolled. Sixty-eight had advanced stage disease, and 80% had B symptoms at diagnosis. After dose-limiting toxicity evaluation of 20 patients, a prespecified stopping criterion was reached and the recommended dose for a phase II trial was 25 mg. At least one grade III/IV toxicity occurred in all 22 patients who were treated at dose levels 20 and 25 mg, and 16 of those patients had a grade IV toxicity. The 1-year estimates for PFS and OS were 69 and 91%, respectively. In summary, AVD-Rev displayed high efficacy and a manageable toxicity profile in older patients with HL and should be further evaluated in phase II/III trials. In addition, a phase II trial combining lenalidomide and panobinostat in patients with relapsed or refractory HL is currently recruiting.

11. Anti-PD-1 Antibodies

The concept that the immune system plays a critical role in controlling and eradicating cancer and that the immune response, driven by T-lymphocytes, is closely regulated through a complicated and delicate balance of inhibitory checkpoints and activating signals is well established [80, 81]. Programmed death-1 (PD-1) is one of the main immune checkpoint receptors that, when binding its programmed death-ligand-1 (PD-L1), determines the downregulation of the T-cell effector functions, thus contributing to the maintenance of the tolerance to tumor cells. The blockade of this pathway by anti-PD-1 and anti-PD-L1 antibodies may prevent this downregulation and allows T-cells to maintain their antitumor property and ability to mediate the tumor cell death [82–84]. The genes encoding the PD-1 ligands, PD-L1 and PD-L2, are key targets of chromosome 9p24.1 amplification, a recurrent genetic abnormality in the nodular sclerosis type of HL. The 9p24.1 amplicon also includes JAK2, and gene dose-dependent JAK-STAT activity further induces PD-1 ligand transcription [85]. The complementary mechanisms of PD-1 ligand overexpression in HL suggest that this disease may have genetically determined vulnerability to PD-1 blockade. For these reasons, in a phase I study, 23 extensively pretreated patients with relapsed or refractory HL were given every 2 weeks 3 mg/kg nivolumab, a fully human monoclonal IgG4 antibody directed against PD-1 [86]. The majority of these patients had previously received an auto-SCT, and most had received previous brentuximab vedotin. Drug-related adverse events of any grade were reported in 18 (78%) of 23 patients, and grade III drug-related adverse events were reported in five (22%) patients. Of 23 patients,

Table 1: Novel agents evaluated in relapsed/refractory HL patients after auto-SCT.

Author	Therapeutic agent(s), study design	Pts. $N1$	Pts. $N2$	Response rate	Median duration of response
Younes et al., 2010 [48]	Brentuximab vedotin, phase I	42	33	ORR = 38% CR = 24%	9.7 months
Younes et al., 2012 [49]	Brentuximab vedotin, phase II	102	102	ORR = 75% CR = 34%	20.5 months for patients in CR
Moskowitz et al., 2013 [55]	Bendamustine, phase II	35	27	ORR = 53% CR = 33%	5 months
Zinzani et al., 2015 [57]	Bendamustine, retrospective	27	27	ORR = 55.5% CR = 37%	8 months
LaCasce et al., 2014 [58]	Bendamustine + brentuximab, phases I-II	45	—	ORR = 94% CR = 82%	NR
Younes et al., 2012 [61]	Panobinostat, phase II	129	129	ORR = 27% CR = 4%	6.9 months
Younes et al., 2011 [65]	Mocetinostat, phase II	51	43	ORR = 33%	NR
Johnston et al., 2010 [68]	Everolimus, phase II	19	16	ORR = 47% CR = 5%	7.2 months
Younes et al., 2012 [72]	Pacritinib, phase I	34	14	ORR = 14%	130 days
Younes et al., 2003 [74]	Rituximab, phase II	22	18	ORR = 22%	7.8 months
Fehniger et al., 2011 [76]	Lenalidomide, phase II	38	33	ORR = 17%	15 months
Kuruvilla et al., 2008 [77]	Lenalidomide, phase II	14	10	PR = 14% SD = 50%	Median OS was 9.1 months
Böll et al., 2010 [78]	Lenalidomide phase II	42	NR	ORR = 50%	NR
Ansell et al., 2015 [86]	Nivolumab, phase I	23	18	ORR = 87% SD = 13%	PFS in 24 weeks was 86%
Moskowitz et al. 2014 [87]	Pembrolizumab, phase I	15	15	ORR = 53% CR = 20%	NR

Pts. $N1$: total number of patients; Pts. $N2$: patients who received prior auto-SCT; ORR: overall response rate; CR: complete remission; PR: partial remission; SD: stable disease; OS: overall survival; PFS: progression-free survival; NR: not reported.

four (17%) had a CR, 16 (70%) had a PR, and three (13%) had SD. In 24 weeks, the rate of PFS was 86% resulting in a very high proportion of patients achieving an overall response and clinical benefit. The study shows promising results; however, larger trials are needed before introducing nivolumab in HL treatment.

A multicenter, open-label, phase Ib clinical trial is ongoing evaluating the use of the humanized IgG4 monoclonal antibody pembrolizumab (formerly MK-3475), targeting the PD-1 receptor, in relapsed or refractory HL patients who failed brentuximab vedotin treatment, with adequate performance status and organ function [87]. Pembrolizumab 10 mg/kg was administered in 15 patients intravenously every 2 weeks until confirmed tumor progression, excessive toxicity, or completion of 2 years of therapy. The drug was well tolerated with no serious adverse events, and only one patient experienced grade III pain and grade III joint swelling. The most common drug-related adverse events were grade I/II respiratory events (20%) and thyroid disorders (20%). Three patients (20%) had a CR in 12 weeks. Five additional patients (33%) had a PR as the best overall response, for an ORR of 53%. Four patients (27%) experienced progressive disease, although all 4 experienced a decrease in their overall tumor burden. In conclusion, pembrolizumab therapy appears to be safe, tolerable, and associated with clinical benefit in patients with heavily pretreated HL.

12. Conclusions

Auto-SCT is the standard of care for refractory/relapsed HL, leading to long-lasting responses in approximately 50% of relapsed patients and in a minority of refractory patients. Patients progressing after intensive treatments, such as auto-SCT, have a very poor outcome.

In the recent past, particularly effective novel therapies have been identified to treat these patients (Table 1). These agents have all been tested as single drugs acting on different pathways implicated in the pathogenesis of HL (Table 2), and therefore an important future approach will be to combine them with each other and with standard chemotherapies.

Up to now, brentuximab vedotin is the only FDA approved drug for the treatment of relapsed HL. There have been attempts to combine brentuximab vedotin in a pretransplant setting, either in sequential mode, that is, brentuximab vedotin as a single agent, followed by HDC, or concurrently (i.e., with bendamustine). Either way, there is an improvement in the overall response rate and complete response rate with these treatment strategies, and this may evolve with time to include brentuximab vedotin as part of the induction in pretransplant regimens.

PD1-targeted therapies, pembrolizumab and nivolumab, are becoming very good potential drugs, and most likely both will be approved in the near future.

Table 2: Competitive environment.

Agent	Indication	Development stage	Mechanism of action
Brentuximab vedotin	HL, NHL	Approved for HL and NHL	Anti-CD30 antibody-drug conjugate
Bendamustine	NHL, MM	Approved for NHL	Bifunctional alkylating agent
Panobinostat	AML, CML, breast cancer, prostate cancer, MM, idiopathic myelofibrosis, HL, NHL	Approved for MM	HDAC inhibitor
Mocetinostat	AML, solid tumors, CLL, MDS, NHL, HL	Phase II	HDAC inhibitor
Everolimus	Solid tumors, transplant rejection, HL, NHL	Approved for solid tumors and transplant rejection	mTOR inhibitor
Pacritinib	AML, myeloproliferative disorders, HL, NHL	Phase III	JAK2-inhibitor
Rituximab	NHL, CLL, rheumatoid arthritis, HL, granulomatosis, multiple sclerosis, MM	Approved for NHL, rheumatoid arthritis, granulomatosis, CLL	Anti-CD20 antibody
Lenalidomide	MDS, MM, NHL, HL, CLL	Approved for MDS, MM	Immunomodulator
Nivolumab	Melanoma, lung cancer, renal cancer, HL	Phase I	Anti-PD1 antibody
Pembrolizumab	Melanoma, lung cancer, renal cancer, HL	Phase I	Anti-PD1 antibody

HL: Hodgkin lymphoma; NHL: non-Hodgkin lymphoma; MM: multiple myeloma; AML: acute myeloid leukemia; CML: chronic myeloid leukemia; MDS: myelodysplastic syndrome; CLL: chronic lymphatic leukemia.

Although these new therapies have clearly demonstrated efficacy in HL, a molecularly targeted drug achieving long-term responses with good tolerability is still lacking. Moreover, the majority of patients are young, and in this scenario we believe that allo-SCT can play an important role in selected patients.

Conflict of Interests

The authors report no conflict of interests or funding sources.

Authors' Contribution

Quality control of data and algorithms has been done by Roberta Fedele, Massimo Martino, Anna Grazia Recchia, Massimo Gentile, and Fortunato Morabito. Paper preparation has been done by Roberta Fedele and Massimo Martino. Paper editing has been done by Roberta Fedele, Massimo Martino, and Anna Grazia Recchia. Paper review has been done by Roberta Fedele, Massimo Martino, Anna Grazia Recchia, and Fortunato Morabito. Approval of the submitted and final versions has been done by Roberta Fedele, Massimo Martino, Anna Grazia Recchia, Massimo Gentile, and Fortunato Morabito. Giuseppe Irrera contributed to paper review and the approval of the submitted and final versions.

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Research Article

Serum CEACAM1 Elevation Correlates with Melanoma Progression and Failure to Respond to Adoptive Cell Transfer Immunotherapy

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Malignant melanoma is a devastating disease whose incidences are continuously rising. The recently approved antimelanoma therapies carry new hope for metastatic patients for the first time in decades. However, the clinical management of melanoma is severely hampered by the absence of effective screening tools. The expression of the CEACAM1 adhesion molecule on melanoma cells is a strong predictor of poor prognosis. Interestingly, a melanoma-secreted form of CEACAM1 (sCEACAM1) has recently emerged as a potential tumor biomarker. Here we add novel evidences supporting the prognostic role of serum CEACAM1 by using a mice xenograft model of human melanoma and showing a correlation between serum CEACAM1 and tumor burden. Moreover, we demonstrate that serum CEACAM1 is elevated over time in progressive melanoma patients who fail to respond to immunotherapy as opposed to responders and stable disease patients, thus proving a correlation between sCEACAM1, response to treatment, and clinical deterioration.

1. Introduction

The incidence of melanoma has more than doubled over the last two decades, making it one of the fastest rising cancers worldwide. When diagnosed at early stages, the disease is curable by surgical removal. Currently, however, the mortality rate is significantly higher than the 1.3% diagnosed with incurable metastatic disease at presentation, implying its metastatic potential (reviewed in [1, 2]). The clinical landscape of antimelanoma drugs has evolved remarkably over the last years by the generation of molecular targeted therapies (BRAF and MEK inhibitors) and immunotherapies (anti-CTLA4 and anti-PD1 antibodies) [3, 4].

The monitoring of melanoma patients relies mainly on physical examination, history taking, periodical imaging, and routine blood tests. There are no simple tests for monitoring melanoma patients in the outpatient setting and the available serum biomarkers (the most reliable and wildly used one being LDH) provide limited information [5, 6]. The rapid rise in melanoma prevalence, emerging era of antimelanoma therapies which are beneficial only for a subset of the patients, and the extraordinary ability of malignant melanoma to remain dormant before relapsing all emphasize the need for novel prognostic biomarkers for melanoma.

CEACAM1, an adhesion molecule belonging to the CEA (carcinoembryonic Ag) family, is a transmembrane glycoprotein expressed on epithelial, endothelial, and hematopoietic cells, where it regulates immune responses, insulin clearance, and neovascularization [7–9]. In healthy volunteers CEACAM1 expression can thus be detected mainly in the luminal side of epithelial cells forming ducts or glands in the visceral organs such as the small intestine, liver bile canaliculi, the kidney, and salivary gland and in hematopoietic cells such as neutrophils [10]. While downregulated in some cancers [11–14], CEACAM1 is elevated stepwise during the course of melanoma progression [15]. Its expression in melanoma

strongly correlates with the development of metastases and poor survival, and its prognostic value is similar or even superior to that of the widely accepted Breslow score (determining tumor thickness at presentation) [16]. While it is expected that the pattern of nonhematological tissue-specific expression of CEACAM1 in melanoma patients would be similar to healthy donors, it has never been directly studied. We have previously shown that an unusual elevated level of CEACAM1-positive T cells and NK cells is found in the circulation of melanoma patients [17] and that CEACAM1 serves as immune evasion mechanism from NK and T cells [18–20]. Based on these findings, we have raised an anti-CEACAM1 blocking antibody that renders melanoma cells more vulnerable to cytotoxic immune cells both *in vitro* and *in vivo* and is a promising strategy for treating melanoma patients [4, 10].

While the therapeutic significance of anti-CEACAM1 therapy awaits further examination, a soluble form of CEACAM1 (sCEACAM1) was described in body fluids, including serum, bile, saliva, and seminal fluid [21–24]. The exact function of sCEACAM1 is still unknown. We reported that the secretion of sCEACAM1 from melanoma cells is an active process, which depends on protein synthesis and intact intracellular vesicular transport, and does not result merely from dead cells or shedding and is not correlated with surface membrane expression intensity [17].

Abnormal decreased levels of sCEACAM1 are found in TAP-2 deficient patients [21]. Elevated sCEACAM1 levels characterize several malignancies, among them are biliary diseases (i.e., obstructive jaundice, primary biliary cirrhosis, autoimmune hepatitis, and cholangiocarcinoma) [22, 24, 25], meningococcal sepsis [26], and, importantly, malignant diseases such as malignant melanoma [17, 27], pancreatic cancer [28, 29], bladder cancer [30], and non-small-cell lung cancer [31]. In melanoma, serum CEACAM1 is elevated in patients with evidence of disease as compared with patients with no evidence of disease or healthy controls, and its expression correlates with LDH, disease state, and decreased survival [17]. Moreover, following vaccination with modified autologous melanoma cells as postsurgical adjuvant therapy, the changes in postvaccination serum CEACAM1 correlate with overall survival and with the S100B melanoma marker [27]. Thus, serum CEACAM1 is a potential novel prognostic biomarker for melanoma progression and predication of response to treatment.

Here we study in xenograft models the correlations between human sCEACAM1 in mice sera and tumor burden, in various scenarios of disease progression, surgical removal of tumor mass, and relapse. The potential of serum CEACAM1 to reflect or predict response to therapy of metastatic melanoma patients with adoptive cell transfer of tumor infiltrating lymphocytes was also tested.

2. Materials and Methods

2.1. Healthy Volunteers and Melanoma Patients. We used a cohort of 47 healthy volunteers. 27 patients were males (57%). 14 donors were <40 years of age, nine were 41–50, thirteen were 51–61, and eleven were 61+ years of age. We used a cohort of 27 AJCC stage IV malignant melanoma patients, showing no other signs of malignancy or health disorders. All patients were treated with tumor infiltrating lymphocytes (TIL) immunotherapy [32, 33] after being refractory to other treatments. All TIL treatments were based on the "young TIL" protocol, except for one patient who was treated with the "selected TIL" protocol [32, 33]. One of the patients was given two sequential TIL treatments with 13 months apart and partially responded to each of them. These treatments were considered as two different sets in our analysis, which consisted of 28 sets of pre- and posttherapy values. Twenty patients were males (74%). Five patients were <40 years of age, seven were 41–50, twelve were 51–60, and three were 61+ years of age.

2.2. Clinical Study Design. A longitudinal retrospective clinical study was performed, in which patients' sera were collected and analyzed for sCEACAM1 levels by ELISA. Samples were obtained in the Sheba melanoma clinic at 3 time points: on decision to go for TIL therapy, which was 43–103 days before TIL treatment (median: 49.5 days), at first posttreatment evaluation which was 48–102 days after treatment (median: 86.5 days), and at second posttreatment evaluation which was 330–552 days after treatment (median: 348 days). Samples were collected from May 2006 through March 2011. The follow-up period was 70–1519 days (median: 194.5 days). The clinical data was analyzed at June 2015. Following evaluation for radiological response, the patients were described according to RECIST 1.0 criteria as progressive disease patients (PD, $n = 10$), stable disease (SD, $n = 6$), or responders that included the partial (PR, 7 patients; 8 data sets) or complete response (CR, $n = 4$) patients. Notably, the evaluations were not changed between the 1st and the 2nd posttreatment time points. All patients gave written informed consent prior to their participation in this study. This study was approved by the Israel Ministry of Health.

2.3. Clinical Samples Handling. Blood samples were obtained from healthy donors and melanoma patients by venopuncture and standard handling procedures. 10 mL of blood was collected in heparinized tubes (BD Biosciences) and then centrifuged at 590 g for 15 min in room temperature to obtain plasma. All plasma samples were collected and divided into aliquots and frozen in −80°C until analyzed. Anonymous samples (marked only with ID number) were linked only to clinical-pathological data.

2.4. Melanoma Xenograft Model. In the xenograft model we use the primary melanoma culture 009mel which was developed from surgically resected tumor and was established and grown as previously described [32]. 3×10^6 009 melanoma cells were injected subcutaneously to the thigh of 7-8-week-old SCID-NOD mice to create human melanoma xenografts. Mice were monitored once weekly for tumor volume by caliper measurements. Tumor volume was calculated as $(\text{small diameter})^2 \times (\text{large diameter})/2$. Mice were sacrificed when showing a reduction of more than 20% in body weight or when tumor volumes reached

3800 mm^3. For tumor excision experiments, 20 mice were used. When tumors reached a volume of 500 mm^3, mice were randomized into 2 equal experimental groups, with one of them undergoing tumor excision (which was complete in 3 mice and partial in 7 mice) and the other sham surgery. All animal work was performed following approval of Sheba Medical Center IRB (861/2013).

2.5. Mice Sera Samples. Blood samples were collected once weekly from the retroorbital plexus of anesthetized mice as described [34]. Anesthesia was induced by placing each mouse in an inhalation chamber with 4% isoflurane (Abbott). The volume of each blood sample was ~250 μL and at no time did this volume exceed that recommended for mice in regard to body weight and recovery time. Mice were allowed to recover completely after each bleeding session and were observed daily for signs of pain and discomfort. Blood samples were deposited in heparinized tubes (BD Biosciences), centrifuged for serum separation (15 min at 590 g at room temperature), and frozen at −80°C until a later, technically convenient point. Serum hemolysis was evaluated by direct observation.

2.6. Anti-Human CEACAM1-Based ELISA. All sera used were thawed at once and subjected to anti-human CEACAM1 ELISA as described [17], using MRG1 as the capture antibody. The anti-human CEACAM1 antibody MRG1 was generated by us as described [10]. Each sample was tested in triplicate repeats.

2.7. LDH Evaluation in Sera Samples. LDH was evaluated in sera samples from 26 out of the 27 patients participating in the cohort, using kinetic UV quantitative evaluation on Beckman Coulters AU analyzers (Beckman Coulter LDH reagent OSR6128). LDH tests were performed on sera samples obtained at the same time points as used for serum CEACAM1 evaluations.

3. Results

3.1. Serum CEACAM1 Correlates with Melanoma Tumor Volume in Xenografted Mice. We have previously shown that CEACAM1 in its soluble form (sCEACAM1) is secreted from several primary cultures and cell lines of human melanoma [17]. The concentration of secreted CEACAM1 was found to be proportional to the number of melanoma cells seeded in culture [17]. In order to test whether serum CEACAM1 correlates with melanoma mass *in vivo* we used a xenograft model in which primary human melanoma cells are injected subcutaneously to SCID-NOD mice [10] and serum CEACAM1 is measured by anti-human CEACAM1-based ELISA. 10 mice were thus injected to the thigh with 3×10^6 009mel cells. Once a week, starting from the day of injection and for 3 weeks, tumor volumes were measured by caliper and sera were collected, frozen, and stored until all samples were gathered and used for ELISA (Figure 1). Notably, murine serum CEACAM1 was not recognizable by the anti-human CEACAM1 antibody ([10] and tumor volume zero point in Figure 1). In contrast, human serum CEACAM1 strongly and directly correlated with tumor burden (Pearson's $R = 0.863$, P value < 0.0001) and was detectable even at minor tumor volume of 14 mm^3, implying its high sensitivity (Figure 1).

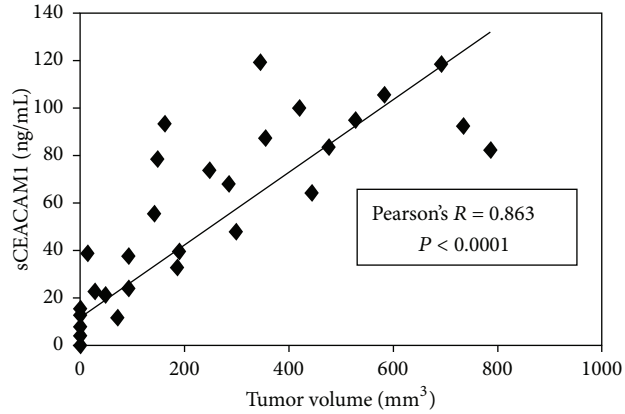

Figure 1: sCEACAM1 directly correlates with tumor burden in mice xenografts. 10 SCID-NOD mice were subcutaneously xenografted with human 009mel. Starting from the day of injection and through 3 weeks, serum samples were collected for further anti-human CEACAM1 ELISA and tumor volume was measured once weekly. The graph shows sCEACAM1 levels versus tumor volume.

3.2. Tumor Excision and Recurrence in Xenografts Are Readily Reflected by sCEACAM1. We next tested whether serum CEACAM1 levels follow the clinical scenarios of tumor excision and recurrence. 20 SCID-NOD mice were injected as described above with 009mel cells. When tumors reached ~500 mm^3, tumor was excised from half of the mice in a complete or nearly-complete manner, while the other half served as a control group and underwent sham surgery. Tumors were measured and sera collected periodically as in Figure 1, except for the week of surgery, in which sera were collected three times from each mouse. Serum CEACAM1 readily followed tumor volumes and dropped dramatically two days after excision. In cases where the excision was complete, serum CEACAM1 gradually vanished from the circulation (Figure 2(a)), whereas in cases where tumor cells remained and tumor recurred, it reincreased in parallel to the elevation in tumor mass (Figures 2(b) and 2(c)). In the sham surgery group, tumors as well as serum CEACAM1 levels continued to increase gradually as expected (Figure 2(d)). We conclude that serum CEACAM1 sensitively and accurately reflects tumor burden in xenografted mice and may therefore serve as a novel biomarker for the monitoring of melanoma tumor burden and progression.

3.3. Baseline sCEACAM1 and LDH Levels Are Higher in Patients Who Fail to Respond to Treatment. In order to test the potential value of serum CEACAM1 for monitoring disease progression and response to treatment in melanoma patients, a retrospective longitudinal clinical trial was performed. We used a cohort of 27 AJCC stage IV melanoma patients that underwent immunotherapy with tumor infiltrating lymphocytes (TIL) [32, 33]. As expected [17, 27], serum CEACAM1 average levels were significantly

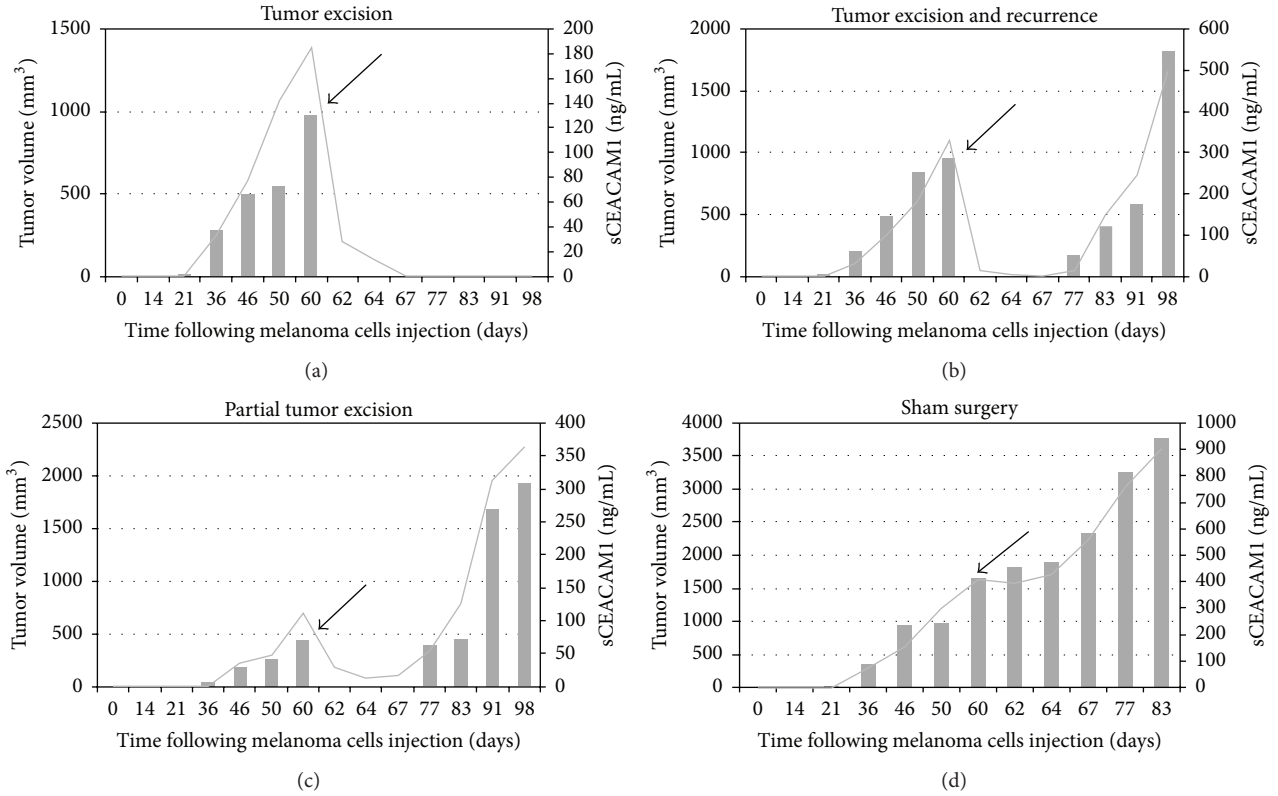

Figure 2: sCEACAM1 sensitively reflects tumor excision and recurrence in mice. 20 SCID-NOD mice were subjected to subcutaneous injection of 009mel cells. When tumors reached ~500 mm^3, they were excised from half of the mice in a complete ((a), $n = 3$) or partial ((b)-(c), $n = 7$) manner while the other mice underwent sham surgery ((d), $n = 10$). In some of the mice recurrence of tumor occurred ((c), $n = 4$). sCEACAM1 levels (depicted by a solid line) as well as tumors volumes (gray bars) were periodically measured and plotted against time. The arrow denotes the excision time point. The experiment was repeated two independent times. Shown are results from one representative mouse from each group.

($P < 0.001$) higher in the whole cohort of melanoma patients as compared with healthy volunteers (Figure 3). In our clinical study, the response to treatment was evaluated starting from 30 days following TIL administration by radiological examination according to RECIST 1.0 criteria. Strikingly, there was a significant difference in the baseline (pretreatment) levels of serum CEACAM1 in between the patients (Figure 4(a), left columns). At this pretreatment time point, serum CEACAM1 was higher by 39% and 34% in patients that were later found to be not responding to treatment (PD) as opposed to responders and stable disease patients (285 versus 204 and 213 ng/mL, resp., $P = 0.03$). Furthermore, 26 out of the 27 patients were assayed for LDH at the same time point. Examining LDH values (Figure 4(b), left columns), we noticed that LDH was significantly elevated in PD patients as opposed to responders but not significantly in PD as opposed to SD patients. Though further examinations with a larger cohort are warranted, this result may point to the prognostic value of serum CEACAM1 in predicting response to adoptive transfer cell therapy or potentially other forms of immunotherapy.

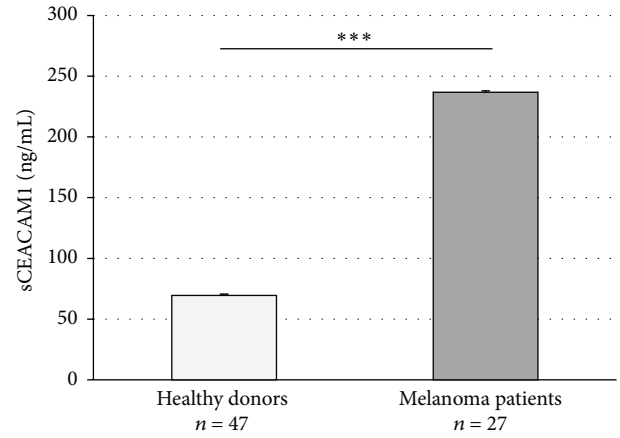

Figure 3: Serum CEACAM1 in healthy donors and melanoma patients. sCEACAM1 levels were assayed in the serum of 47 normal, healthy volunteers and in 27 melanoma patients. Statistics were assayed by regular Student's t-test.

3.4. sCEACAM1 and LDH Levels Are Elevated following Therapy in PD Patients Only. We continued by examining the changes in serum CEACAM1 and LDH following adoptive transfer cell therapy at 2 time points: 1.5–3.5 months (median: 86.5 days) and 11–18 months (median: 348 days) after

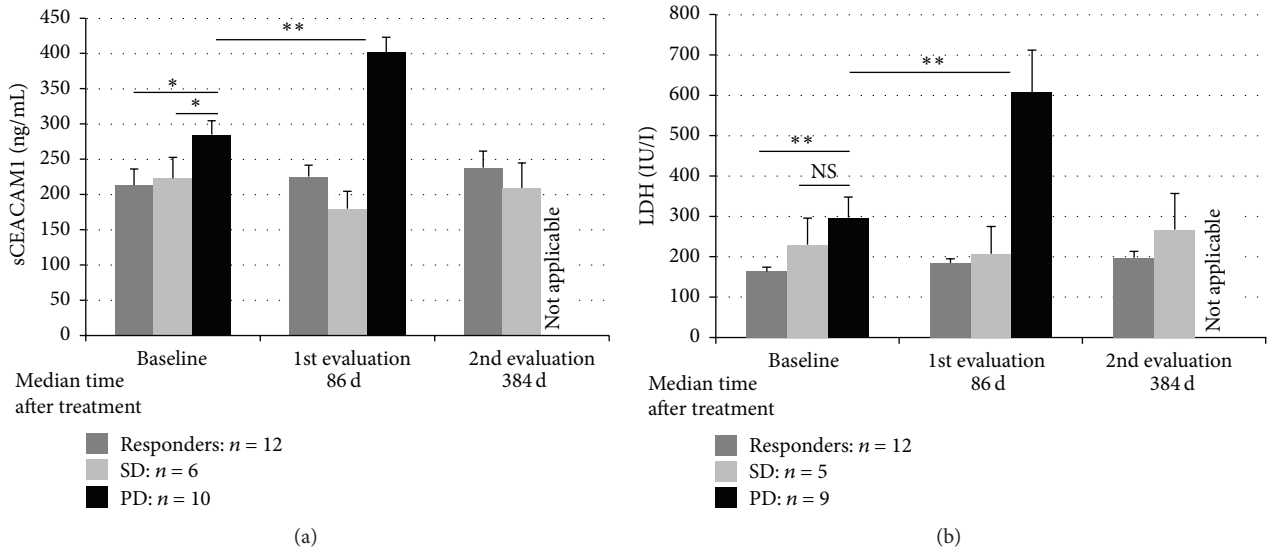

Figure 4: sCEACAM1 and LDH are elevated in melanoma patients who fail to respond to immunotherapy. sCEACAM1 (a) and LDH (b) levels were measured in clinically derived serum samples of AJCC stage IV metastatic melanoma patients that underwent immunotherapy with tumor infiltrating lymphocytes before as well as at 2 points after treatment (except for PD patients who passed away before the later time point). Statistics were assayed by regular Student's t-test.

TIL administration (Figures 4(a) and 4(b), middle and right columns). Unfortunately, PD patients passed away before the second posttreatment checkpoint could be achieved. We found that in patients who manifested disease progression (PD patients) serum CEACAM1 was significantly ($P = 0.001$) increased following treatment (a 40% increase, from 285 to 401 ng/mL in ~4 months). These results are in line with the xenograft evidences on the direct correlation between serum CEACAM1 and tumor burden. As expected, LDH values also increased following treatment (from 297 to 607 IU/I, $P = 0.005$). In the other groups of patients that did not show clinical deterioration, that is, the SD patients and the responders (CR and PR), there was no significant change in serum CEACAM1 (Figure 4(a)) or LDH (Figure 4(b)) over the ~17-month follow-up period.

3.5. The Changes in sCEACAM1 following Therapy Correlate with Those in LDH. We continued by assessing the relationships between serum CEACAM1 and LDH in response to TIL immunotherapy. We chose to relate to the 1st posttreatment values, which were available for all patients' subgroups, and assayed the changes in serum CEACAM1 (ΔsCEACAM1) and LDH (ΔLDH) at this time point as compared to baseline levels. Importantly, assessing the changes by the nonparametric Spearman correlation, we found a significant (Spearman coefficient = 0.764; significance 2-tailed = 0) correlation between ΔLDH and ΔsCEACAM1 (Figure 5 and Table 1).

Another parameter we tested is the time intervals from baseline to the 1st posttreatment time points, which ranged in between patients (baseline: 43–103 days before TIL treatment with median of 49.5 days and 1st posttreatment point at 48–102 days after treatment with median of 86.5 days). Specifically, we asked whether the differences in time intervals between patients (Δdays) could affect

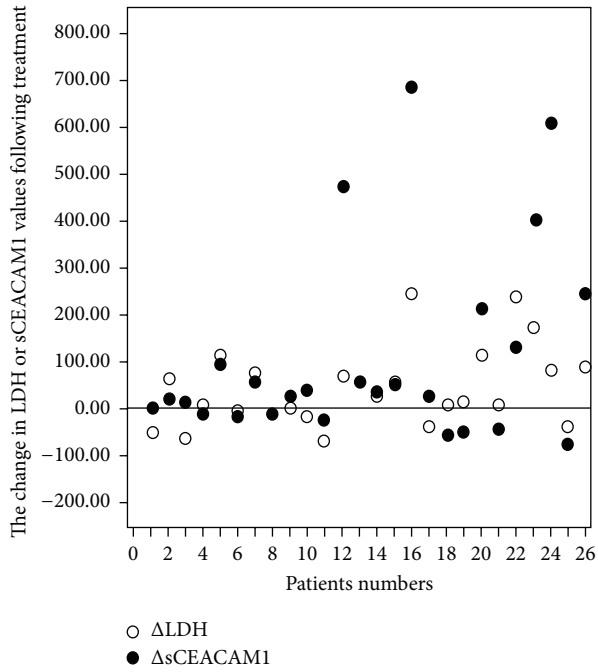

Figure 5: The changes in serum CEACAM1 in patients responding to treatment correlate with those in LDH. sCEACAM1 and LDH levels were tested at the same time points before and following immunotherapy for 26 AJCC stage IV patients. The baseline, pretreatment levels of sCEACAM1 (or LDH) were then subtracted from posttreatment levels and plotted as black (sCEACAM1) or white (LDH) circles for each of the patients.

the obtained ΔsCEACAM1 and ΔLDH values. Using Spearmen correlation, we found that ΔLDH and ΔsCEACAM1 were not correlated with Δdays (i.e., were not increased as

Table 1: The correlations between ΔsCEACAM1, ΔLDH, and Δdays in melanoma patients.

	ΔLDH versus ΔsCEACAM1	ΔLDH versus Δdays	ΔsCEACAM1 versus Δdays
Correlation coefficient	0.764	0.19	0.202
Significance (2-tailed)	0	0.354	0.323

Spearman nonparametric correlation was used to assess the relationships between the changes of sCEACAM1 and LDH following treatment and between the differences in time intervals of testing points between patients and each of the markers.

Δdays was increased; Table 1), supporting the strength of our findings.

Altogether, the results presented in this study imply a direct correlation between elevation in serum CEACAM1 levels, disease progression, and response to treatment and strengthen the prognostic value of sCEACAM1 in melanoma.

4. Discussion

Despite recent progression in the field of antimelanoma therapies, the prognosis of malignant melanoma patients still presents a clinical challenge as reliable biomarkers are scarce. LDH, which is mainly secreted from dead or damaged cells (concurring with tumor burden), is the strongest predictive serological marker for melanoma. It is incorporated in the TNM melanoma staging together with tumor thickness, mitotic rate, ulceration, and the presence of metastases [35–37]. Other reported candidate biomarkers include VEGF, tyrosinase, osteopontin, YKL-40, S100B, IL-8, and Cox-2. These, however, are also manifested by normal cells and under other malignancies (infectious disease, liver and renal injuries, autoimmunity, etc.) and therefore manifest undesired level of false-positive readouts [38–44]. Moreover, the existing markers are not suitable for deciphering specific subsets of patients and dictating therapeutic choices, such as patients who will benefit from novel immuno- and targeted therapies (and their combinations) or early-stage patients who are at high risk of relapse [1]. Therefore, urged by the generation of novel therapies, there is mandatory need for the discovery of melanoma biomarkers. Here we focused on a novel emerging melanoma biomarker, serum CEACAM1.

In this work we demonstrate that serum CEACAM1 sensitively reflects tumor volume in mice xenografted with human melanoma (Figures 1 and 2). This is in line with our previous results that serum CEACAM1 correlates with melanoma cell number in culture [17]. Importantly, serum CEACAM1 could be detected in mice even at minimal tumor volume of 14 mm^3 which may imply on its prognostic value as an early diagnosis of melanoma. Indeed, it was shown that the changes in sCEACAM1 in non-small-cell lung cancer patients are more pronounced in early than in advanced tumors [31]. Moreover, in a large prospective study on pancreatic cancer patients, sCEACAM1 was found to be one of the earliest to be detected at significantly altered levels up to 35 months prior to diagnosis [28].

We have previously shown in two independent retrospective clinical studies that serum CEACAM1 is significantly higher in melanoma patients from different AJCC stages who show evidence of disease at the time of sampling, as compared to patients with no evident disease and healthy volunteers [17, 27] (Figure 3). Moreover, we found that serum CEACAM1 inversely correlates with survival [17, 27] and can stratify melanoma patients with evidence of disease into two prognostic groups with different survival rates [17]. In this study we continued using a different clinical scenario and explored AJCC stage IV melanoma patients that were subjected to adoptive cell transfer immunotherapy with tumor infiltrating lymphocytes (TIL ACT) after being refractory to all other treatments. We found that in patients who have not responded to treatment and continued to manifest progressive disease serum CEACAM1 was significantly ($P = 0.001$) higher as compared with responders and stable disease patients (Figure 4(a)). Collectively, these results show that serum CEACAM1 reflects tumor burden, disease progression, and survival.

Notably, serum CEACAM1 has not changed in responders following treatment in the two time checkpoints tested (Figure 4(a)). It may be that the expected decrease in sCEACAM1 in these patients was masked by CEACAM1 that was secreted from other cells so that the net serum CEACAM1 levels were balanced. Indeed, serum CEACAM1 is secreted from normal cells and is readily detected in the sera of healthy volunteers [17]. Moreover, it was demonstrated that apoptosis could induce cleavage of the intracellular and extracellular domains of CEACAM1, resulting in an increased level of serum CEACAM1 [45]. It may also be that at later time points, which were not checked, a change in serum CEACAM1 could be observed in responders.

Interestingly, when examining the baseline levels of serum CEACAM1, which were measured ~86 days before treatment, we found that serum CEACAM1 was significantly ($P = 0.03$) higher in PD patients, who fail to respond to treatment, as opposed to SD and responders (Figure 4(a), baseline). Noteworthy, in our experimental set-up, the same patients did not exhibit a statistically significant difference in LDH values in between PD and SD patients (Figure 4(b), baseline). We tested a battery of cytokines, including IL-8, TNFα, MIP1, MCP1, IL-4, and IL-17a, in pretreatment (basal) serum samples of melanoma patients who responded to TIL treatment ($n = 8$) as compared with patients who failed to respond ($n = 8$). Unfortunately, no significant differences were found between the groups in all cytokines tested (data not shown). These results point on possible prognostic value of serum CEACAM1 in predicting response to immunotherapy.

Collectively, our results show the prognostic value of serum CEACAM1 in monitoring tumor burden and disease progression. Though additional studies in larger cohorts and various therapeutic scenarios are warranted, they imply on the possible importance of serum CEACAM1 in early

detection of melanoma and in prediction of response to immunotherapy.

5. Conclusions and Clinical Relevance

In this study we demonstrate that serum CEACAM1 (sCEACAM1) levels are correlated with tumor burden in immune-deficient mice xenografted with human melanoma. Moreover, in a clinical retrospective study ($n = 28$) we show that sCEACAM1 is increased only in patients who failed to respond to adoptive cell transfer therapy with tumor infiltrating lymphocytes and manifested progressive disease deterioration (PD). Strikingly, these patients were characterized by a higher pretreatment sCEACAM1, as compared with SD and responders. Moreover, the changes in post- versus pretreatment sCEACAM1 correlate with those in LDH. Altogether, these results imply on the prognostic value of sCEACAM1 in monitoring tumor burden, disease progression, and response to immunotherapy.

Conflict of Interests

Gal Markel is the Chief Scientific Officer of cCAM Biotherapeutics. The rest of the authors declare no conflict of interests.

Authors' Contribution

R. Ortenberg and S. Sapoznik have equally contributed to the work. J. Schachter and G. Markel have equally contributed to the work.

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Research Article

In Vitro and In Vivo Comparison of Lymphocytes Transduced with a Human CD16 or with a Chimeric Antigen Receptor Reveals Potential Off-Target Interactions due to the IgG2 CH2-CH3 CAR-Spacer

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The present work was designed to compare two mechanisms of cellular recognition based on Ab specificity: firstly, when the anti-HER2 mAb trastuzumab bridges target cells and cytotoxic lymphocytes armed with a Fc receptor (ADCC) and, secondly, when HER2 positive target cells are directly recognized by cytotoxic lymphocytes armed with a chimeric antigen receptor (CAR). To compare these two mechanisms, we used the same cellular effector (NK-92) and the same signaling domain (FcεRIγ). The NK-92 cytotoxic cell line was transfected with either a FcγRIIIa-FcεRIγ (NK-92^{CD16}) or a trastuzumab-based scFv-FcεRIγ chimeric receptor (NK-92^{CAR}). In vitro, the cytotoxic activity against HER2 positive target cells after indirect recognition by NK-92^{CD16} was always inferior to that observed after direct recognition by NK-92^{CAR}. In contrast, and somehow unexpectedly, in vivo, adoptive transfer of NK-92^{CD16} + trastuzumab but not of NK-92^{CAR} induced tumor regression. Analysis of the in vivo xenogeneic system suggested that the human CH2-CH3 IgG2 used as a spacer in our construct was able to interact with the FcR present at the cell surface of the few NSG-FcR+ remaining immune cells. This interaction, leading to blockage of the NK-92^{CAR} in the periphery of the engrafted tumor cells, stresses the critical role of the composition of the spacer domain.

1. Introduction

The clinical benefits associated with adoptive immunotherapy of some mAbs have established the clinical pertinence of several antigens as immune therapeutic targets. For some therapeutic antibodies such as the anti-CD20 rituximab or the anti-HER2 trastuzumab, cell-mediated immunity (antibody-dependent cellular cytotoxicity (ADCC)) has been recognized as one of the mechanisms responsible for their clinical efficiency [1, 2]. Accordingly, several strategies have been considered to increase the ADCC potential in patients [3]. Cellular subsets capable of mediating ADCC include neutrophils, monocytes/macrophages, and a subset of natural killer (NK) cells. As an example of a strategy to improve patient ADCC potential, we have shown in the context of breast cancer patients that their NK population could be amplified in vitro up to 425-fold using a straightforward culture procedure [4]. Yet, because of technical limitations associated with the clinical use of NK cells (poor recovery after freezing and thawing, poor in vitro expansion compared to T cells) and the numerous approaches that have become available to engineer lymphocytes (see for review [5]), we have also considered the possibility of arming T cells with a receptor that would enable them to mediate ADCC [3].

In the above study, we have shown that, after transduction with FcγRIIIa/FcεRIγ (referred to as CD16/γ) receptor fusion gene, CD4+ and CD8+ cytotoxic T lymphocytes displayed a stable expression of the CD16/γ receptor at their surface and mediated ADCC. Thus, associating a therapeutic mAb and an adoptive transfer of CD16/γ transduced T cells could combine the advantages associated with the functional potential of cytotoxic lymphocytes and recognition of the target cells unrestricted by the major histocompatibility complex. Another way to reach the same objective is to redirect T cells with the so-called chimeric T cell receptor (CAR) or "T bodies," a strategy pioneered more than two decades ago by the team of Eshhar [6–8], which has recently shown impressive antitumor effects in patients with hematologic diseases (for a review see Gill and June [9]). These are fusion proteins between single chain variable fragments (scFv) from a monoclonal antibody and an intracellular signaling domain such as CD3ζ or FcεRIγ. The above two strategies will be referred to in the text as the ADCC approach (a treatment relying on an mAb + an adoptive transfer of CD16 armed T cells) and the CAR approach (the adoptive transfer of CAR armed T cells). Both strategies have the same fundamental advantage, which is the combination of the cellular immune potential of T cells with recognition of a target cell unrestricted by the MHC. But they also differ on several clinical, immunological, and practical aspects. In particular, in terms of safety, as highlighted by Morgan et al. in their in-depth analysis of a serious adverse event following the administration of T cells transduced with a CAR against HER2 [10], a major difference between mAb administration and adoptive transfer of CAR engineered T cells is that while Ab is cleared by the body, CAR T cells can continuously produce effector cytokines and can expand in cell numbers following antigen stimulation. This may be a real advantage or a real danger, depending on the tumor restriction of the antigen targeted. Provided that the tumor antigen targeted is appropriately restricted, the main advantage of the CAR approach would be its efficiency. On the other hand, the ADCC approach allows the search of a correct dosage simply by changing the dose of the mAb. The aim of the present study was to directly compare the CAR and ADCC approaches with a clinically relevant target antigen. To this end, as a first step in designing a model, we equipped the same cytotoxic lymphocyte line (the human NK cell line NK-92) with either a FcγRIIIa/FcεRIγ receptor (referred to as NK-92^{CD16}) or an anti-HER2/FcεRIγ CAR receptor (referred to as NK-92^{CAR}) and compared their efficiency in killing HER2 positive tumor target cells in vitro and in vivo. While in vitro comparison between these two effectors has highlighted an advantage in the CAR approach in terms of cytotoxic potency, the in vivo experiments performed in NSG mice have not enabled a CAR/ADCC comparison but instead have revealed an off-target interaction which blocked the potential antitumoral efficacy of the CAR modified lymphocytes.

2. Results

2.1. Expression of the Chimeric Anti-HER2 Receptor (CAR) and the CD16 on the Surface of NK-92. The chimeric cDNAs were synthesized by GeneCust (Dudelange, Luxembourg). The CD16/γ chimeric cDNA comprises the leader (S) and the two extracellular domains (EC1 and EC2) of human CD16^{H48V158} and two amino acids (aa) of the extracellular domain of the human FcεRIγ (Pro4-Gln5), as well as the intact transmembrane (TM) and intracellular (IC) domains (Figure 1(a)). The trastuzumab-based CAR contains the VL and VH from the mAb (Ab4D5-8), separated by a linker, the human CH2-CH3 IgG2 as a spacer, and the same signaling domain as that of CD16 (Figure 1(a)). After transduction (see Section 4), 41% of the NK-92 expressed CD16 and 36% expressed the CAR. After immunomagnetic purification using anti-CD16 and anti-human IgG2a-Fc-specific mAb (see Section 4), essentially pure populations of NK-92^{CD16} and NK-92^{CAR} were obtained (Figure 1(b)).

2.2. In Vitro Comparison of HER2-Specific Cytotoxicity Mediated by NK-92^{CD16} + Trastuzumab or NK-92^{CAR}. First, we showed that the spontaneous cytotoxic activity (tested against K562, E/T ratio: 30/1) of the untransduced and the transduced NK-92 was not significantly modified by the retroviral transduction (Figure 2(a)). Next, ADCC activity of the NK-92^{CD16} against the BT474 cell line was tested in the presence of increasing concentration of trastuzumab (as in the example shown in Figure 2(b)) and demonstrated a plateau (close to 50% specific lysis) at 10 μg/mL (E/T ratio: 30/1). No ADCC was observed in the presence of rituximab and NK-92^{CD16} or NK-92^{NT}. Note that a background lysis was observed for NK-92^{NT} + trastuzumab (compared to NK-92^{NT} + rituximab), likely due to background expression of CD16 by the NK-92 cell line. For all other in vitro experiments, trastuzumab was used at a concentration of 10 μg/mL. Next, we compared the efficiency of target cell lyses induced either after direct recognition of the HER2 Ag by the NK-92^{CAR} alone or after indirect recognition by the NK-92^{CD16} in the presence of trastuzumab. To this end, target cells (the HER2 negative MDA-MB-468 and the HER2 positive BT474) were coated or not with trastuzumab, and the untransduced NK-92^{NT} was used as a negative control. Cytotoxic activities of NK-92^{CAR} and NK-92^{CD16} against trastuzumab coated or not coated MDA-MB-468 and BT474 are summarized in Figure 2(c) (at an effector-to-target ratio of 30 : 1). Neither NK-92^{CAR} nor NK-92^{CD16} presented a significant level of cytotoxicity against the HER2 negative MDA-MB-468 cell line (Figure 2(c), left panel). When tested against the BT474, NK-92^{CAR} showed a high level of cytotoxic activity (101 ± 3% at 30 : 1). Killing of BT474 was also observed by NK-92^{CD16} in the presence of trastuzumab, although this occurred at a level below that observed by NK-92^{CAR} (46 ± 8% at the same effector-to-target ratio of 30 : 1). In addition, the preincubation of the BT474 in the presence of trastuzumab drastically reduced the killing by NK-92^{CAR} (Figure 2(c), right panel). To confirm the differences in cytotoxic performance between NK-92^{CD16} and NK-92^{CAR}, further comparison was performed against 4 different HER2 positive cell lines: BT474, BT474 scid (a subclone of BT474 used for the in vivo experiments), MCF7, and MDA-MB-231 (Figure 3). These data showed that, in

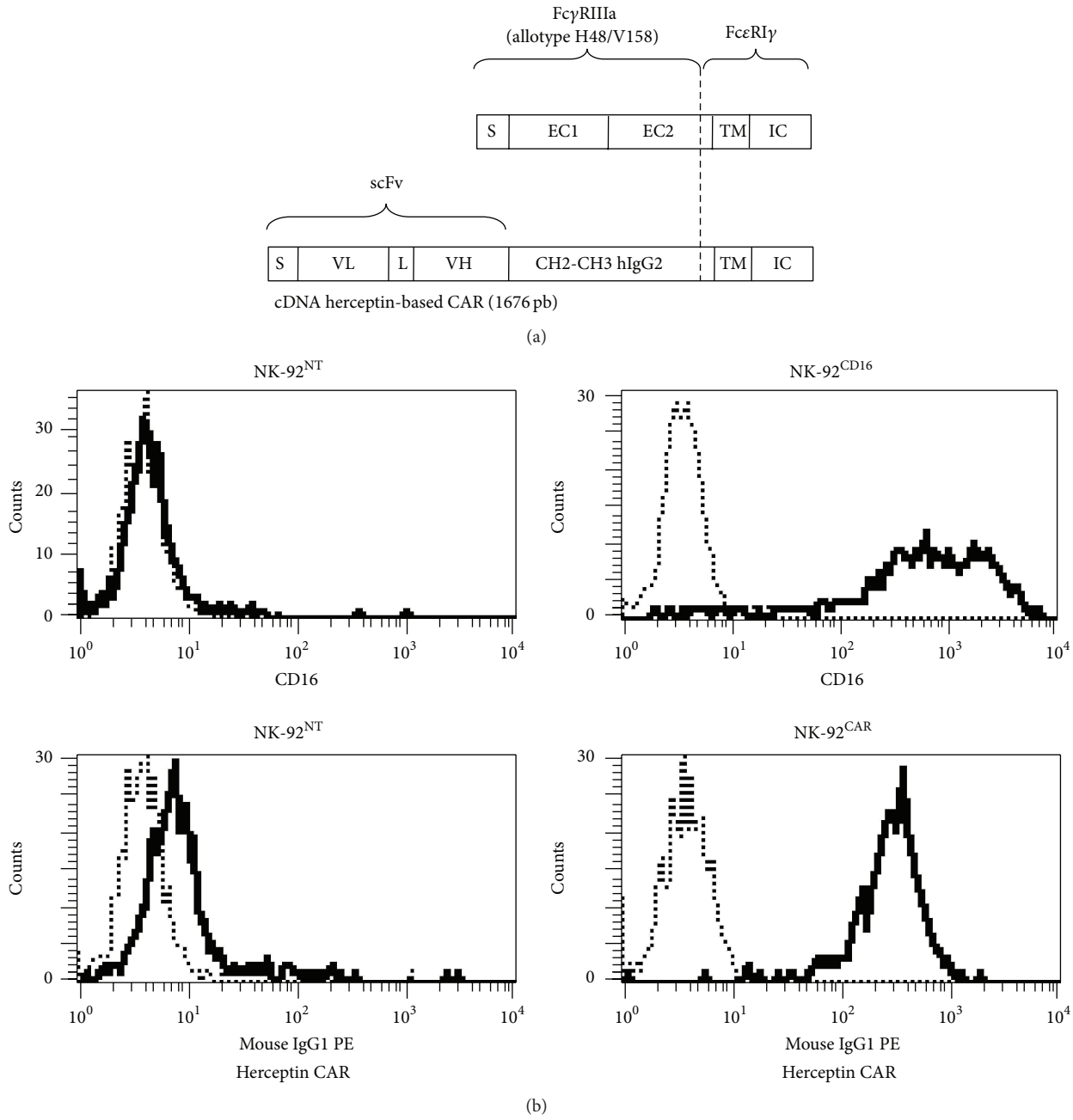

Figure 1: CD16 and CAR vector design and expression in NK-92 cells. (a) Schematic representation of the chimeric human FcγRIII-human FcεRIγ molecule and the trastuzumab- (4D5-) based CAR against HER2: the CD16/γ chimeric cDNA comprised the leader (S) and the two extracellular domains (EC1 and EC2) of human $CD16^{H48V158}$, two amino acids (aa) of the extracellular domain of human FcεRIγ, and the intact transmembrane (TM) and intracellular (IC) domains. The trastuzumab- (4D5-) based CAR contains the VL: hum Ab 4D5-8 light chain; a linker; VH: hum Ab 4D5-8 heavy chain and two amino acids (aa) of the extracellular domain of the human FcεRIγ; and the intact transmembrane (TM) and intracellular (IC) domains. (b) Transgene expression of NK-92 cell line transduced with CD16-γ chimeric receptor or trastuzumab-γ CAR. Anti-CD16 (clone 3G8) was used to determine CD16 expression on CD16-γ transduced NK-92 cell line (solid line), and an isotype Ab was used as negative control (dotted line). Anti-human IgG2a-Fc (clone HP6002) was used to determine trastuzumab-γ CAR expression on trastuzumab-γ CAR transduced NK-92 cell line (solid line), and an isotype Ab was used as negative control (dotted line).

vitro, in these experimental conditions, based upon 4 hr cytotoxicity assays, the direct pathway of killing (by $NK\text{-}92^{CAR}$) was always more efficient than the indirect pathway (by $NK\text{-}92^{CD16}$) (Figures 3(a), 3(b), 3(c), and 3(d)).

2.3. In Vivo Comparison of $NK\text{-}92^{CD16}$ + Trastuzumab and $NK\text{-}92^{CAR}$ to Control the Growth of Established BT474 Tumor. Having shown that $NK\text{-}92^{CAR}$ were more effective in vitro than $NK\text{-}92^{CD16}$ against several HER2 positive tumor cells,

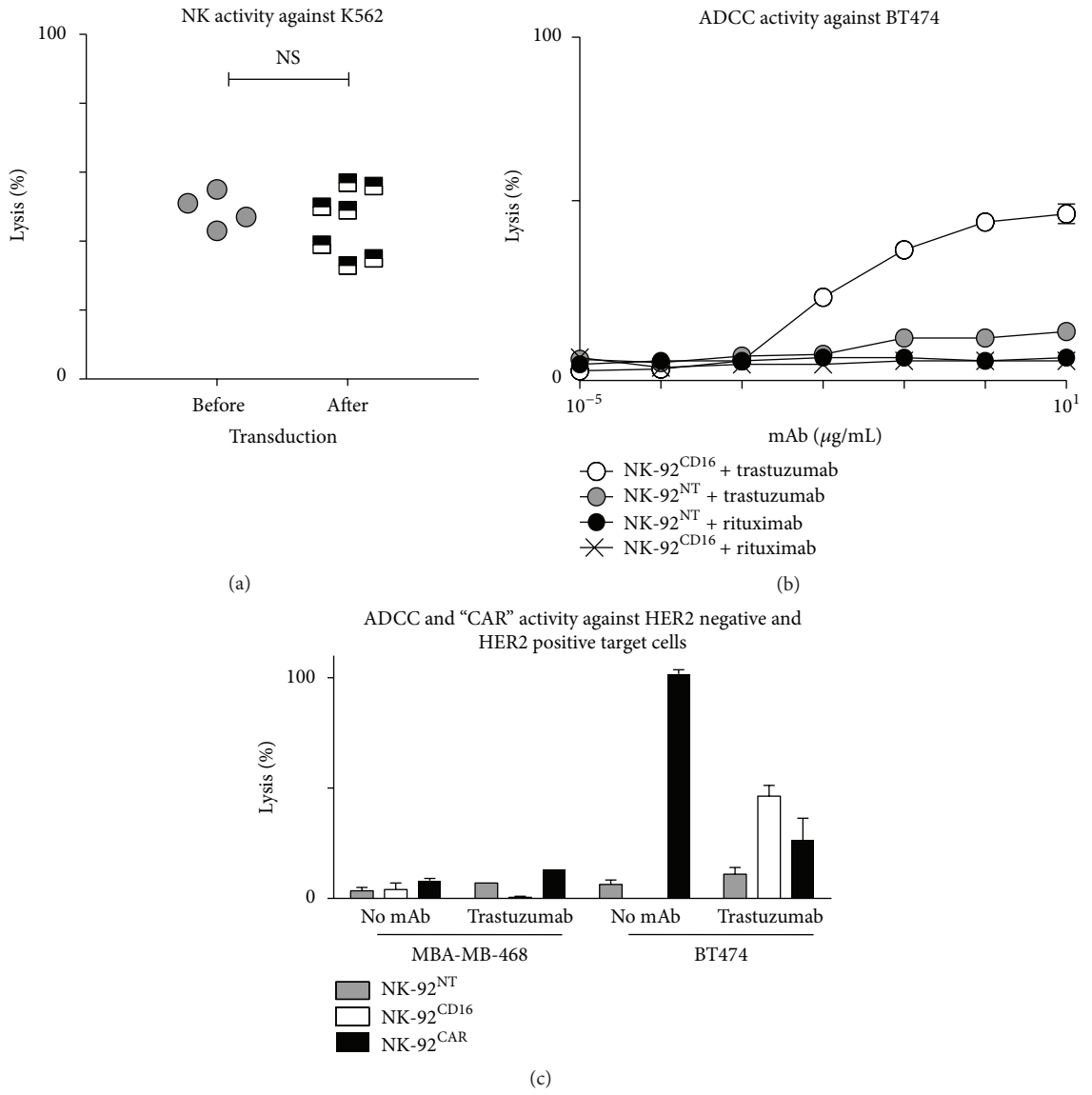

Figure 2: Cytotoxic activity of NK-92^{CD16} and NK-92^{CAR}. (a) Spontaneous (NK) activity of NK-92 before and after transduction (E/T ratio: 30/1). (b) The effector cells NK-92^{CD16} and NK-92^{CAR} were tested against the HER2 positive BT474 cell line preincubated in the presence of increasing concentration of trastuzumab (mean of two experiments). (c) Cytotoxic activity of NK-92^{NT}, NK-92^{CD16}, and NK-92^{CAR} and against HER2 negative (MBA-MB-468) or HER2 positive (BT474) cell lines (trastuzumab, 10 mg/mL, E/T ratio: 30/1). Cytotoxicity was evaluated from ^{51}Cr release after 4 hours of incubation. The data represent the means from three independent experiments.

we developed a mouse xenograft model to test whether such difference could also be evidenced in vivo. Six-week-old female NSG mice were injected subcutaneously in the left side with 5×10^6 BT474 cells, and when the tumor reached a volume of 50 mm^3, the mice received the indicated treatment (all mice were sacrificed before the tumor volume reached 2500 mm^3). We first tested the capacity of IP injection of NK-92^{CD16} and trastuzumab to control the growth of a subcutaneous established NK-92 resistant BT474 carcinoma (Figure 4(a)). In the absence of addition of effector cells, weekly injections of trastuzumab (15 mg/kg) alone do not prevent BT474 tumor burden (Figure 4(a)). When 5×10^6 NK-92^{CD16} cells were used in combination with trastuzumab (15 mg/kg) injected 24 h prior to NK cells, complete tumor regression was observed. Note that tumor regression began after a single dose but that complete regression required 4 injections once per week of 5×10^6 NK-92^{CD16} cells (Figure 4(a)). To confirm that ADCC was the mechanism responsible for tumor regression, experiments were repeated with the untransduced NK-92^{NT} or with NK-92^{CD16} in the presence of an irrelevant antibody (rituximab, directed against CD20, an Ag not present at the surface of BT474). As the result of a mechanism of tumor destruction due to ADCC, neither the untransduced NK-92^{NT} in the presence

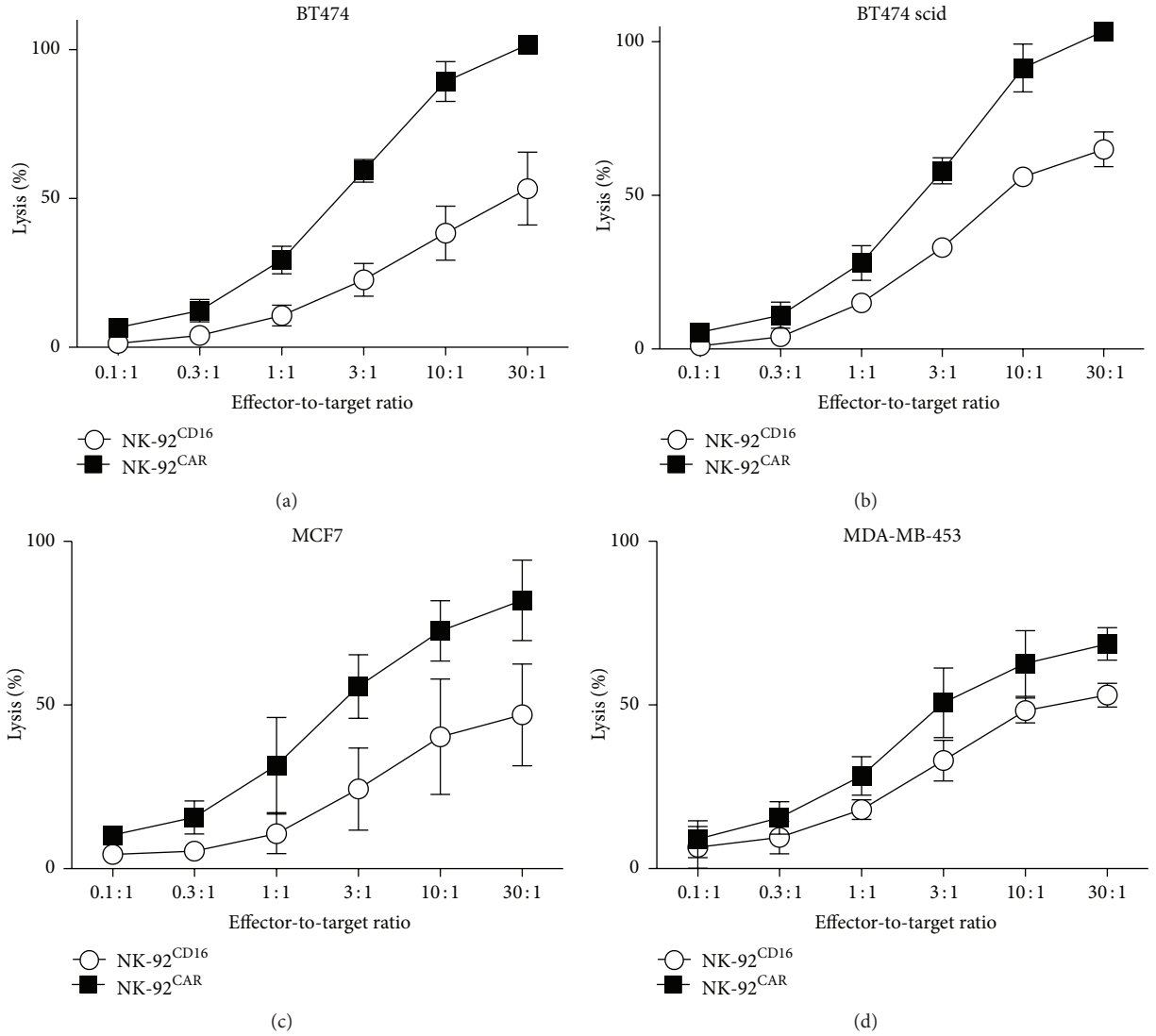

Figure 3: Comparison of direct NK-92^{CAR} or indirect NK-92^{CD16} recognition of 4 HER2 positive cell lines. Cytotoxicity was evaluated from ^{51}Cr release after 4 hours of incubation; the data represent the means of three independent experiments. For ADCC, cell lines were incubated in the presence of 10 μg/mL trastuzumab. In the absence of antibody BT474, MDA-MB-453 and MCF7 were not sensitive to NK activity of NK-92 (see Figure 2 and data are not shown for MCF7).

of trastuzumab nor NK-92^{CD16} in the presence of rituximab were able to control the growth of the BT474 tumor (Figures 4(b) and 4(c), resp.).

We then analyzed the antitumor effect of NK-92^{CAR} in this BT474 xenograft model. In contrast to the in vitro situation, where NK-92^{CAR} has demonstrated a higher efficacy of BT474 killing compared to NK-92^{CD16} + trastuzumab, no tumor regression was observed even after 4 injections with NK-92^{CAR} (Figure 4(d)). Before investigating in more detail the possible reasons for the discrepancy between in vitro and in vivo results, we first controlled the injection route and reproduced the experiment. With NK-92^{CAR} IV instead of IP injection, we observed the same inefficacy (data not shown).

2.4. NK-92^{CAR} Scattered into All Organs but Did Not Spread within the Tumor. To investigate the unexpected total lack of in vivo efficacy of NK-92^{CAR}, we compared the dissemination abilities of NK-92^{CAR} and NK-92^{CD16} in the NSG mouse model. For this purpose, 2×10^7 NK-92^{NT}, NK-92^{CAR}, or NK-92^{CD16} cells were injected into NSG mice bearing BT474 tumors (tumor size ranging between 50 and 100 mm^3). Twenty-four hours later, mouse blood, liver, tumor, lung, and spleen were collected, and the percentages of CD45 positive cells were analyzed by FACS analysis using specific Ab. As shown in Figures 5(b), 5(c), and 5(d), no major differences between NK-92^{NT}, NK-92^{CAR}, and NK-92^{CD16} spreading were found within blood, spleen, and liver, suggesting no major differences in their ability to recirculate within the NSG mice after IP injection. However, whereas NK-92^{NT} and NK-92^{CD16} can be found in tumors even at a low but significant ratio, no NK-92^{CAR} could be found inside the tumor (Figure 5(a)). We then analyzed whether preinjection

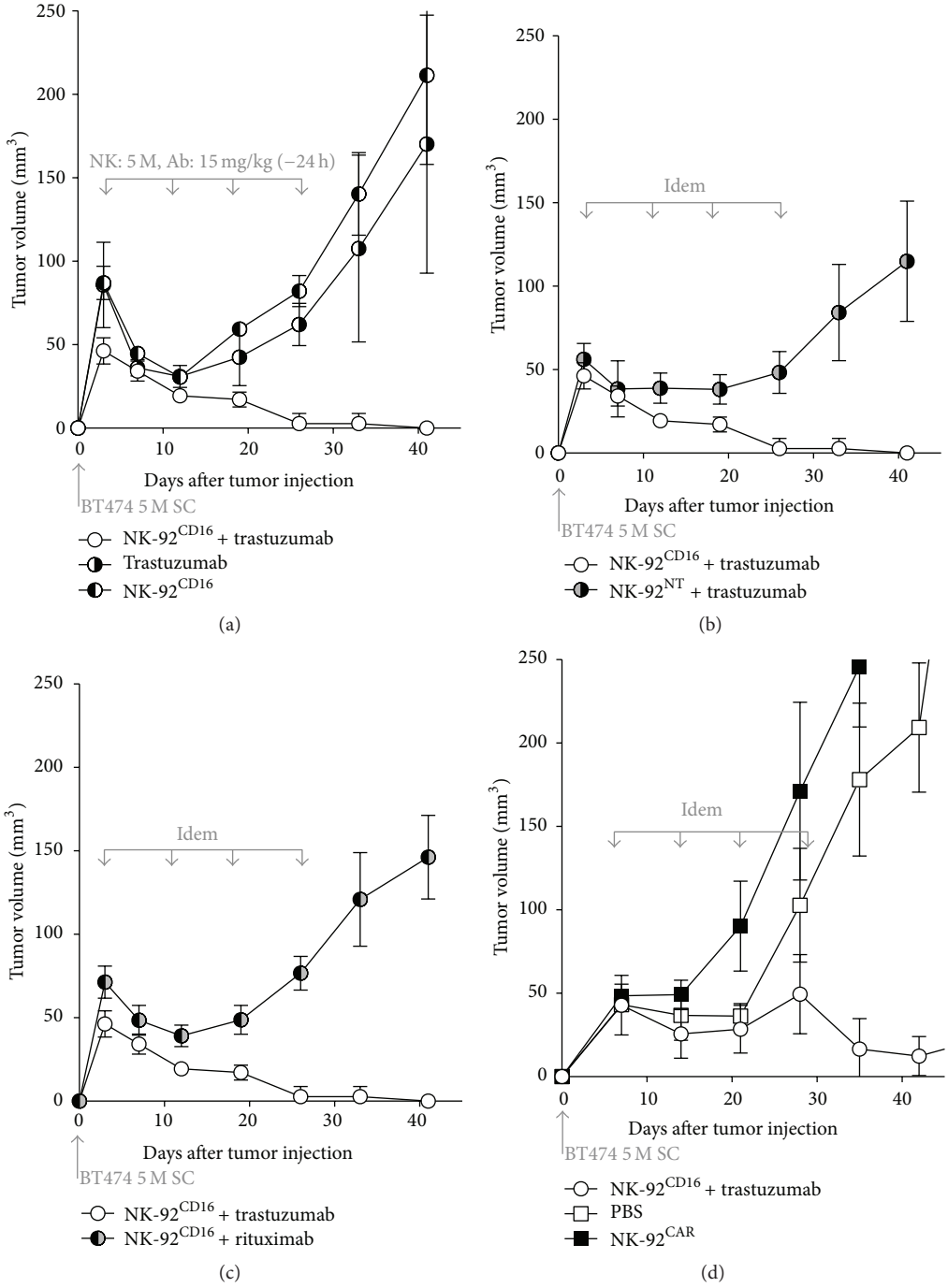

Figure 4: The NK-92^{CD16} in the presence of trastuzumab but not NK-92^{CAR} induces regression of the HER2 positive BT474 tumor engrafted in NSG mice. Results presented in (a), (b), and (c) are from the same series of experiments but are presented separately for readability: the group of mice treated with NK-92^{CD16} + trastuzumab is thus the same for (a), (b), (c), and (d). Six-week-old female NSG mice were injected subcutaneously in the left side with 5×10^6 BT474 cells. When tumors reached a minimal volume of 50 mm^3 (within 5–7 days), mice were individually identified and randomly assigned to the control or treated groups (5 to 15 mice per group) and the indicated treatments were initiated.

of either trastuzumab, competing for HER2 binding with CAR-HER2, or Cetuximab, recognizing HER1 also expressed at the surface of the BT474 cells without interfering with CAR-HER2 binding, could induce a chemoattractive signal for NK cells inside the tumor. As shown in Figure 5(a), opsonization of tumors with specific Ab does not attract NK-92^{CAR} effectors. To confirm these data, we injected 3×10^7 CSFE labelled NK-92^{NT}, NK-92^{CAR}, or NK-92^{CD16} cells into NSG mice bearing BT474 tumors (tumor size ranging between 50 and 100 mm^3) and collected the tumors 72 hours

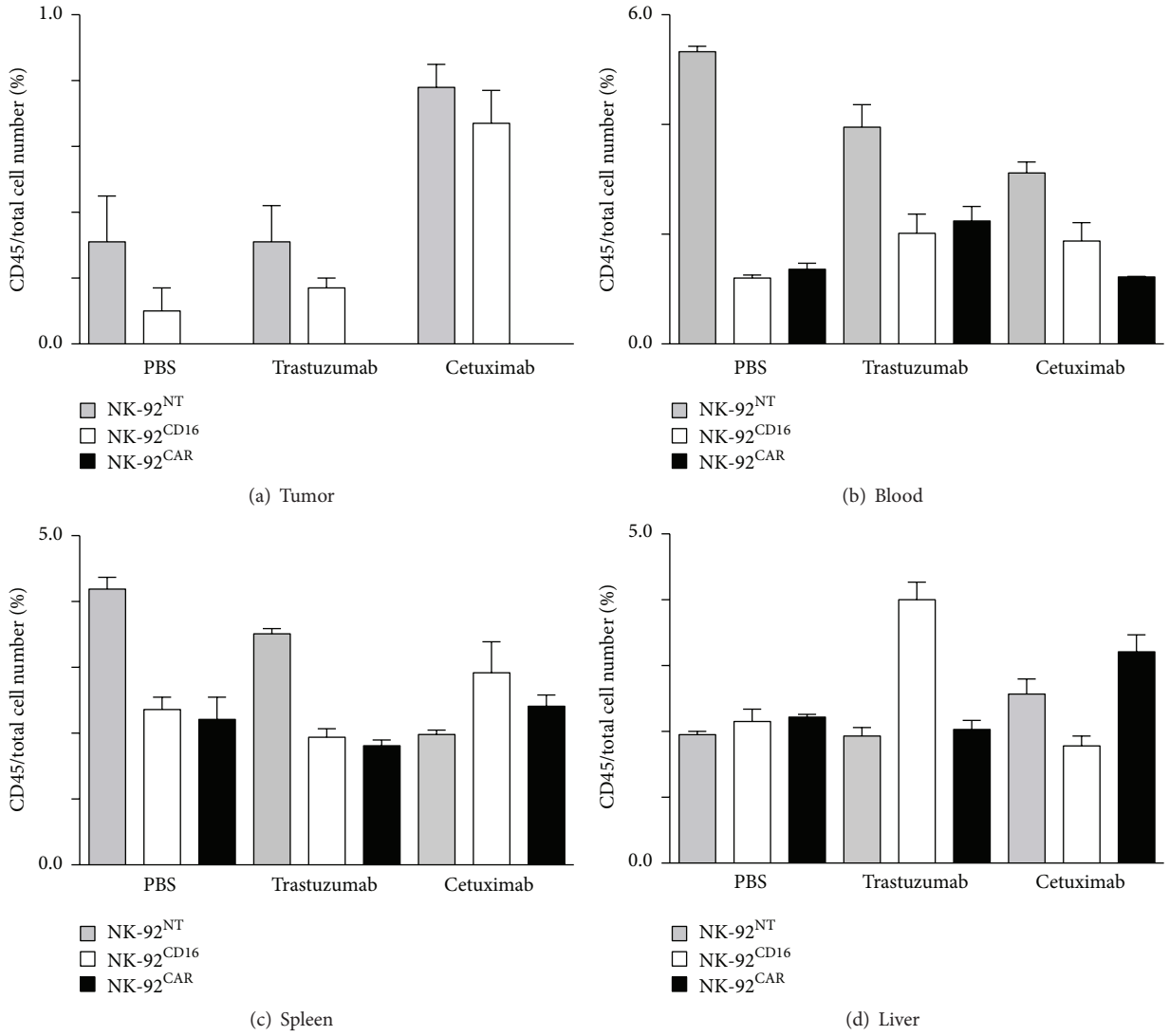

Figure 5: Tissue distribution of NK-92^{NT}, NK-92^{CD16}, and NK-92^{CAR} in NSG mice bearing an established BT474 tumor. NK-92^{NT}, NK-92^{CAR}, or NK-92^{CD16} CSFE labelled cells (20×10^6) were injected into NSG mice bearing BT474 tumors (tumor size range of 50 to 100 mm^3). Twenty-four hours later, mouse blood, liver, tumor, lung, and spleen were collected, and the percentages of CD45 positive cells were analyzed by FACS analysis (percentage of CD45 was calculated from 50,000 events, and the means of two independent experiments are presented).

later. We then performed IHC on frozen sections by direct reading of CFSE labelled NK effectors and visualization of the tumor cell nuclei by counterstaining with DAPI. As shown in Figure 6(a), NK-92^{NT} and NK-92^{CD16} could be found in different parts of the tumors, either as a cluster or as single cells. Preinjection of trastuzumab did not affect the NK-92^{CD16} frequency within the tumor (Figure 6(a), panels 2 and 3). In support of the previous FACS analysis, NK-92^{CAR} effectors could never be found within the tumors. We observed that where present at all, most NK-92^{CAR} were blocked at the edge of the tumor in the form of clusters (Figure 6(b), right panel) whereas NK-92^{CD16} could be found everywhere inside the tumor (Figure 6(b), left panel). Suspecting a possible interaction between mice macrophages and NK-92^{CAR}, we counterstained our frozen IHC sections with anti-Iba1 antibodies specific to mouse macrophages (Wako). As shown in Figure 6(c) right panel, a yellow merge staining always located at the edge of the tumor with NK-92^{CAR} was observed, suggesting that mice macrophages (in red) could aggregate with NK-92^{CAR} effectors (in green). This was never observed inside the tumor, or with NK-92^{CD16} for which isolated macrophages are distributed everywhere inside the tumor (Figure 6(c), left panel, red arrow). Taken together, these data suggest that mice macrophages aggregating NK-92^{CAR} might have prevented them from infiltrating inside the tumor, explaining the lack of efficacy for these type of effectors.

2.5. In Vitro Specific Interaction between NK-92^{CAR} and FcR$^+$ NSG Splenocytes. As shown in Figure 5(a), 24 h after injection, NK-92^{NT} and NK-92^{CD16} are present within the

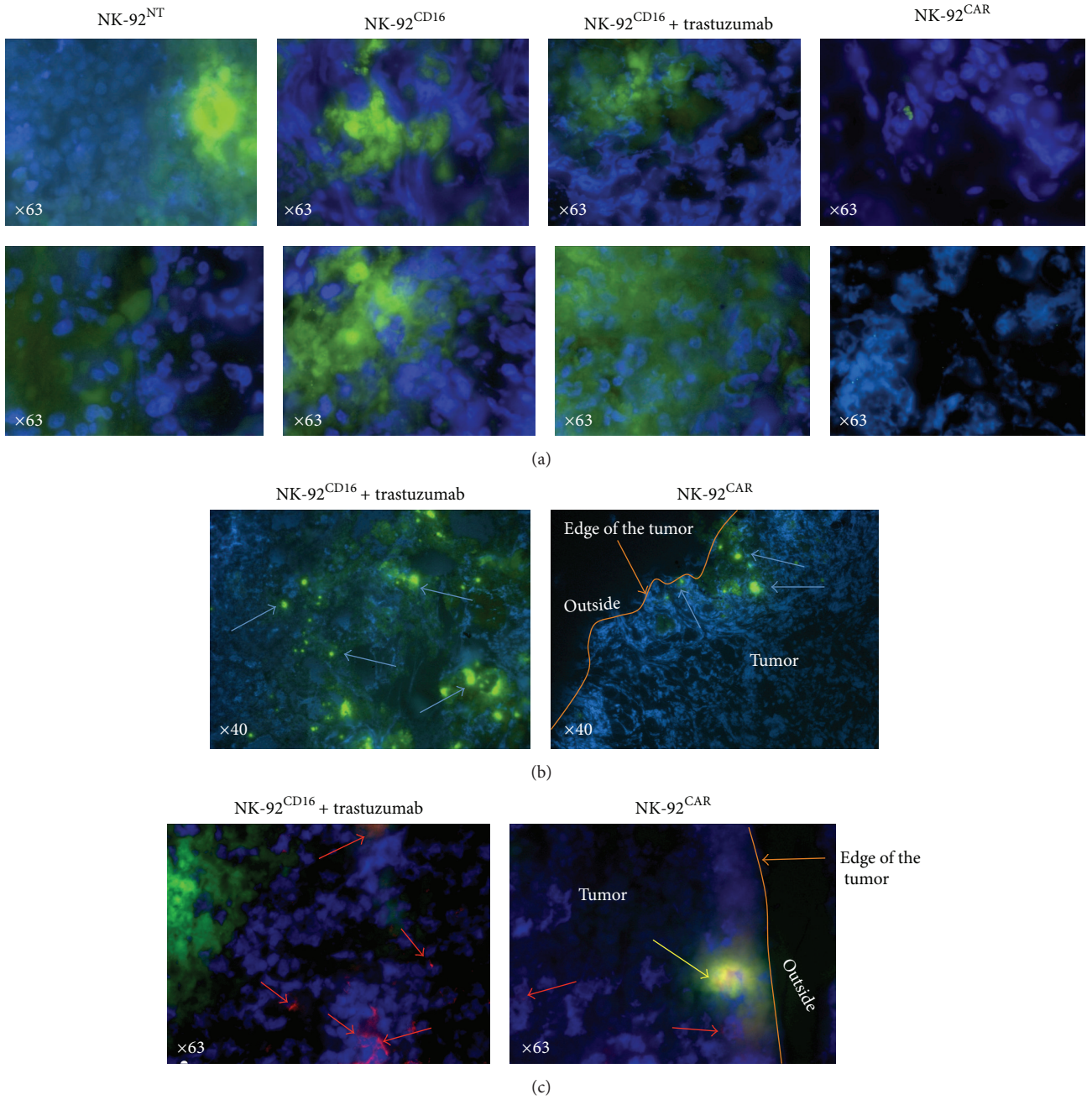

Figure 6: Immunohistochemical analysis of BT474 tumor sections. NK-92 were directly stained with CSFE and tumor cells were visualized by counterstaining with DAPI. (a) NK-92^{NT} and NK-92^{CD16} could be found in different parts of the tumors, either as a cluster or as single cells and preinjection of trastuzumab did not affect NK-92^{CD16} frequency within the tumor (panels 2 and 3). Panel 4 shows the absence of NK-92^{CAR} within the tumors. (b) Comparison of effectors spreading between NK-92^{CD16} and NK-92^{CAR} within the tumors using direct CFSE visualisation (green): NK-92^{CAR} appeared clustered at the edge of the tumor only whereas NK-92^{CD16} are observed everywhere in the tumor. (c) Merge colocalization (yellow arrow) of mice macrophages (red) using Iba1 staining and NK-92^{CAR} cells (green) are always located at the edge of the tumor (blue) whereas isolated macrophages (red arrow) can be found everywhere within the tumor. This was not observed with NK-92^{CD16}. Typical representative images are presented.

tumor, but not NK-92^{CAR}. This was observed whether or not the mice were pretreated with trastuzumab. Because pretreatment of the mice with trastuzumab should have blocked recognition of cross-reactive mice antigens (if any), the interaction between mice macrophages and the CAR was unlikely to be due to the specific VH-VL part of the CAR. In contrast, although NSG mice lack T cells, B cells, and natural killer cells, they nevertheless still have neutrophils, monocyte/macrophages, and dendritic cells. And although these cells are functionally defective because of the

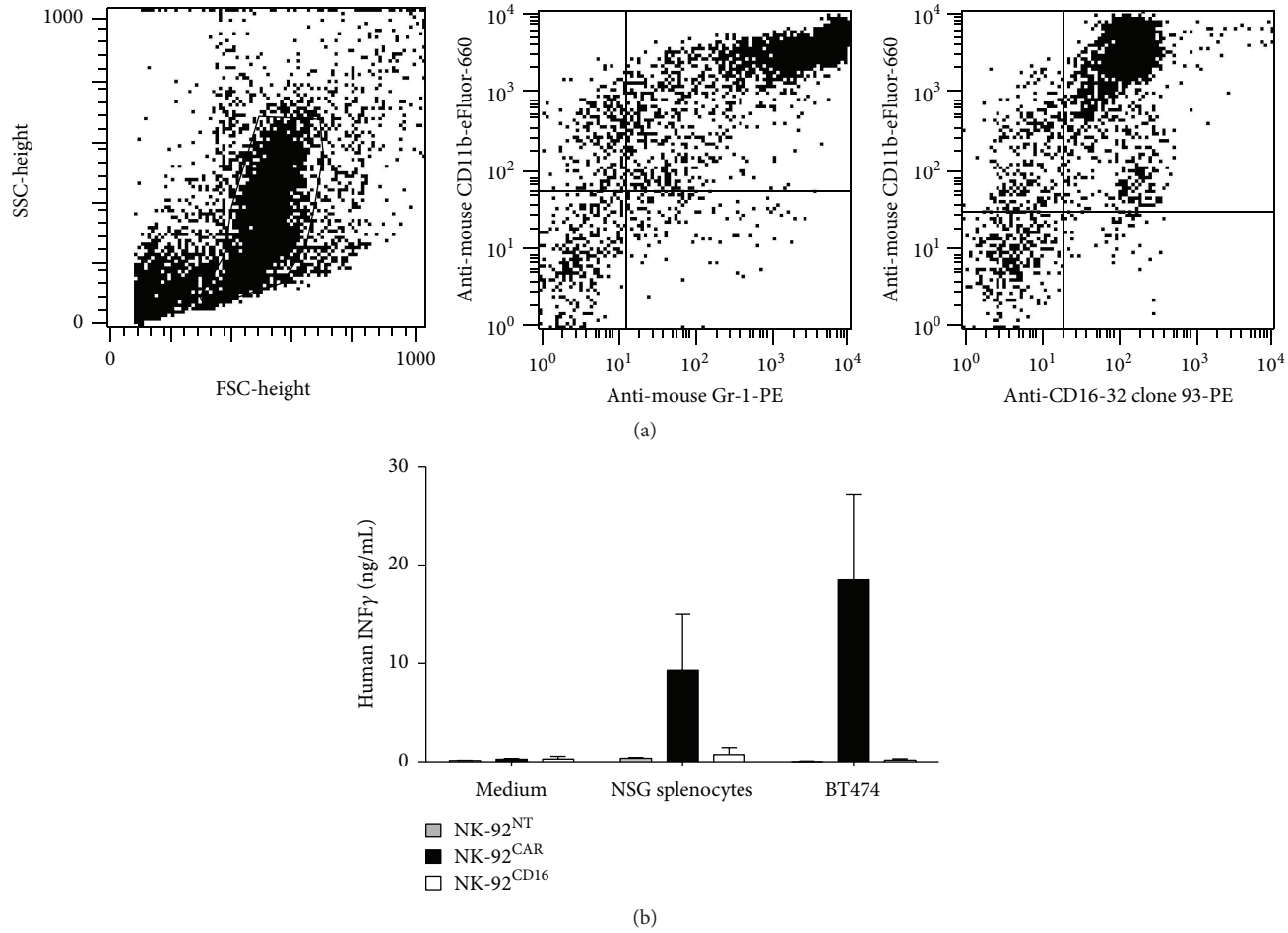

Figure 7: NK-92^{CAR} stimulation after coculture in the presence of NSG splenocytes. (a) Single-cell suspensions from NSG mice spleens were stained with an antibody cocktail and then analyzed by flow cytometry for expression of CD11b (Mac-1) versus Gr-1 (Ly-6G) and CD11b versus CD16/32 (FcRγRIII/FcRγRII). (b) NK-92 (NK-92^{NT}, NK-92^{CD16}, or NK-92^{CAR}) and splenocytes from NSG mice or the HER2 positive cell line BT474 were cocultured at an effector ratio of 5 : 1 and 2 : 1, respectively, in 96-well round bottom culture plates in RPMI 1640 supplemented with 10% FCS and IL2 (100 IU/ML). After 18 h, supernatants were collected and human INF-γ content was estimated using an ELISA kit (Affymetrix, e-biosciences). Data represent the mean with SD from three independent experiments.

NOD/ShiLt genetic background, they nevertheless bear Fc receptors (as shown in Figure 7(a), the CD16-32 staining of the CD11b+/Gr-1+ myeloid NSG splenocytes). And mice Fc receptors can cross-react with the human Fc (see Section 3).

To directly address the possibility of interaction between NSG Fc-bearing cells and the NK-92^{CAR}, NSG splenocytes were cocultured with NK-92^{NT}, NK-92^{CAR}, or NK-92^{CD16} at a 5 : 1 ratio. After 18 hours, NK-92 cell activation was analyzed by quantifying human IFNγ in supernatant by ELISA. As shown in Figure 7(b), coincubation of NSG splenocytes with NK-92^{NT}, NK-92^{CAR}, or NK-92^{CD16} led to detectable production of IFNγ only by NK-92^{CAR}. This production level is far from being insignificant as it corresponds to half of the IFNγ level produced by NK-92^{CAR} after coincubation with the HER2 BT474 tumor cells (9.3 ng/mL and 18.4 ng/mL, resp.). Taken together, these results strongly suggest that NK-92^{CAR} not only have been physically prevented from penetrating the tumor due to being trapped by the surrounding macrophages but also were functionally activated by this off-target interaction.

3. Discussion

We compared in the present study two constructs allowing us to implement two strategies relying on an adoptive transfer of cytotoxic lymphocytes to improve the targeting of HER2 positive tumors. In the first, the lymphocytes are equipped with a human CD16 receptor (FcγRIIIa/FcεRIγ fusion protein) to permit ADCC in the presence of trastuzumab. In the second, we used a first generation CAR encompassing scFv derived from trastuzumab and a CH2-CH3 hIgG2 spacer between the scFv and the transmembrane domain. In both constructs, the signaling domain was the FcεRIγ.

After transduction with CD16 and CAR receptor genes, the human NK cell line NK-92 displayed stable cell surface expression of CD16 and CAR receptors. Specific cytotoxic

activity against HER2 positive target cells was demonstrated in both cases, and when we compared their potency against four HER2 positive target cell lines, NK-92^{CAR} always performed better than NK-92^{CD16}.

To our knowledge, only two studies have previously compared these two approaches directly. Boissel et al., using the two different therapeutic monoclonal anti-CD20 mAbs rituximab and ofatumumab, demonstrated that the cytotoxic activity of NK-92 cells expressing CD20-targeting first-generation CAR against primary CLL cells was superior to the ADCC by NK-92^{CD16} in the presence of anti-CD20 monoclonal antibodies [11]. And Tassev et al., in their detailed description of the retargeting of NK-92 cells using an HLA-A2-restricted EBNA3C-specific chimeric receptor (a TCR-like antibody), also directly compared the two systems and conclude that "the CAR mediated approach proved far more effective at killing target cells compared with ADCC…" [12].

To what extent can these observations be generalized? For ADCC, the outcome of effector/target cell interactions will depend on the number of CD16 receptors, their affinity with the antibody used to recognize the target, the number of target antigens, nonspecific interactions between the target and effector (e.g., LFA-1/ICAM1), time, concentration of Abs in the liquid phase, and so forth. Similarly for CAR recognition, CAR density, CAR affinity, and antigen expression level will all influence the outcome of the interaction. In addition, for CAR, other variables will also be important, such as the choice of transducing chain, or the addition of an accessory signal (such as the 4-1BB).

In the present study as well as in the study undertaken by Boissel et al. and Tassev et al., because the effector was a clone cell line, the only difference in every effector/target interaction was the way target cells were recognized: either by CD16 + Ab or by the corresponding CAR. Thus, these three studies suggest that the ranking CAR > ADCC remains true (i) against different target antigens (and in particular CD20 and HER2), (ii) with scFv of different affinities (rituximab and ofatumumab have an affinity with the CD20 antigen of approximately 5.45 and 4.76 nM, resp. [13], while the (EBNA clone 315) scFv has an affinity of 291 nM with the HLA-A2/EBNA complex [12]), (iii) with different signaling domains, that is, CD3ζ or FcεRIγ, and (iv) with or without the costimulatory signaling domain 4-1BB.

We then attempted to implement a xenogeneic model to continue the comparison in vivo. In NSG mice, we found that IP injection of NK-92^{CD16} and trastuzumab induced total regression of established subcutaneous BT474 tumors (at least until day 40). But in contrast to already published results [14], NK-92^{CAR} that we designed had no antitumor activity, in vivo in NSG. Further analysis demonstrated that, in contrast to NK-92^{CD16}, NK-92^{CAR} did not reach the tumor and were aggregated at the periphery of the tumor by the mice macrophages. In addition, in vitro coincubation of NK-92^{CAR} but not NK-92^{CD16} in the presence of NSG splenocytes led to activation and IFN-γ production. An important difference between NK-92^{CAR} and NK-92^{CD16}, as well as between NK-92^{CAR} used in the present study and that used by Schönfeld et al., was the spacer. For NK-92^{CAR}, we used a CH2-CH3 IgG2 hinge, no hinge was used for the NK-92^{CD16}, and in their NK-92^{CAR} (also against HER2) Schönfeld et al. used the CD8α. Such spacer is sometimes required in the design of a CAR, for the following reasons.

Beyond the variables cited previously, a further specific level of complexity is associated with the use of CAR. The CARs, formerly called "T bodies" by their inventor E. Eschar, can be seen as a sort of TCR having the specificity of an antibody. The TCR recognizes MHC-peptide complexes; these are a unique class of antigens which are characteristic of the exposition at the target cell surface and their physical accessibility to the TCR is homogeneous. The size of a TCR molecule is thus naturally adapted to "sense" the peptide/MHC complex exposed in the target cell. In the same way, the size of the CD16 is naturally adapted to catch the Fc of the Ab bound to the target cell surface, though in this case with more variability than for the TCR, since the exposition of the Fc will depend on the position of the epitope recognized. In nature, this difficulty is solved by the flexibility of the Ab, which may derive from specific characteristics of both the Fab and the Fc region (for a review, see [15]). Consequently, while the steric constraints associated with the target cell recognition by a lymphocyte equipped with a TCR or a CD16 receptor are essentially "natural," this is not the case for a CAR, whose ability to catch the Ag and transmit the signal will rely in part on the characteristics of the CAR hinge region.

Indeed, the distance of the epitope to the target cell surface, as well as the flexibility and length of the CAR hinge region, matters in regard to CAR recognition. In line with the spatial constraints cited above, it has been shown that a protruding hinge region is needed for efficient activation of lymphocytes armed with a CAR recognizing a membrane-proximal epitope, whereas lymphocytes equipped with a CAR specific for a membrane-distal epitope can be efficiently engaged without extracellular spacer element [16, 17].

As many others before us [17–22], we used an IgG hinge region because at first glance it presented many advantages. First, in line with spatial constraints, the IgG Fc domain can provide both flexibility and the possibility of different lengths by adapting the number of CH2 or CH3 molecules; for clinical applications, it lacks immunogenicity; and for detection, it allows the use of anti-Fc reagents. In addition, we made the choice to use the human IgG2 as a spacer domain in our CAR design because of its lower affinity for the human FcγRs [23]. Furthermore, replacement of the IgG1 CH2 sequences with those of IgG2 was shown to eliminate in vitro the activation of CAR T cells by human FcγR-bearing cells and simultaneous cross-activation of cytokine production by innate immune cells [19].

Clearly, this precaution was not enough. CARs are designed to be used in humans and their study in xenogeneic models may reveal specific traps. In particular, the receptors for the Fc domain of IgG, FcγRs, are quite dissimilar in binding abilities and expression pattern between human and mouse [24]. Mice have three activating FcγRs (mFcγRI, mFcγRIII, and mFcγRIV) and one inhibitory FcγRIIb. In

NSG mice, neutrophils and monocytes constitute most of the remaining detectable mouse immune cells. Neutrophils express two activating FcγRs: FcγRI and FcγRIV. Dendritic cells and macrophages are also present in the NSG mouse and although they are functionally defective because of alleles in the NOD/ShiLt genetic background, they express activating FcγRs. Dendritic cells express FcγRI and macrophages express all the FcγRs, and even though they are not functional, they could nevertheless interact with the spacer derived from human IgGFc. Moreover, human IgG2 can bind to the murine FcγRIIb and FcγRIII and induce a potent ADCC with mouse NK cells and mouse polymorphonuclear leukocytes [25]. Finally, the absence of antitumoral activity of NK-92^{CAR} with the human full-length IgG2 Fc derived spacer observed in the present study supports and extends the results obtained using CD19-CAR designed with a full-length human IgG4 Fc spacer, which failed to eradicate Raji tumors in NSG mice unless the entire CH2 domain responsible for FcR binding was removed [22].

In conclusion, our results extend the number of target antigens for which the CAR approach performs better than the ADCC approach in vitro in terms of cytotoxic activity. Moreover, after the recent work of Hudecek et al. [22], we provide additional evidence stressing the potential dramatic effect in vivo of the spacer domain of CARs, even those devoid of intrinsic signaling capacity.

4. Materials and Methods

4.1. Cell Lines. NK-92, the human NK cell line (ATCC, Rockville, MD), was grown in RPMI 1640 culture medium (Gibco, Cergy Pontoise, France) supplemented with 10% FBS (PAA Laboratories, Les Mureaux, France), 100 IU/mL IL-2 (Proleukin) (Chiron Corporation, Emeryville, US), 2 mM L-glutamine (Gibco), penicillin (100 IU/mL), and streptomycin (0.1 μg/mL) (Gibco). Epstein-Barr B-lymphoblastoid cell lines (BLCLs) were derived from donor peripheral-blood mononuclear cells (PBMCs) by in vitro infection with EBV-containing culture supernatant from the Marmoset B95-8 cell line (ATCC) in the presence of 1 μg/mL cyclosporin-A. The HER2 negative MDA-MB-468 and the HER2 positive BT-474, MDA-MB-231, and MCF7 breast cancer cell lines were obtained from ATCC. Cell lines were cultured in complete medium consisting of DMEM (Sigma Aldrich, St. Quentin Fallavier, France), 10% heat-inactivated foetal calf serum, 2 mM glutamine (Sigma Aldrich), 100 U/mL penicillin, and 10 μg/mL streptomycin (Sigma Aldrich).

4.2. Flow Cytometry. Expression of the trastuzumab- (4D5-) based CAR construction against HER2 and of the human CD16 (FcγRIIIa/FcεRIγ) on the surface of NK-92 was determined by direct immunofluorescence using anti-CD16 (clone 3G8) and anti-human IgG2a-Fc that recognize the CH1-CH2 spacer of the CAR (clone HP6002), respectively. For staining 0.1×10^6 cells (untransduced NK-92^{NT}, NK-92^{CD16}, and NK-92^{CAR}) were incubated for 15 minutes at room temperature at the indicated mAb concentrations diluted with PBS supplemented with 0.1% human albumin in a final volume of 30 μL. After staining, plates were centrifuged, the supernatant was discarded by flicking, and wells were washed twice with 200 μL ice-cold PBS. Negative controls were set up in the presence of a control isotype in case of direct staining or in the absence of first Abs for indirect staining. In case of indirect staining, cells were washed after the first incubation and the second Ab was used at saturating concentration.

4.3. Retroviral Vector Production. Transient retroviral supernatants were produced by $CaCl_2$ precipitation with 15 μg of plasmid. Two million Phoenix-Ampho cells [26] were seeded into 10 cm diameter dishes 24 h prior to transfection. The transfection was performed with 15 μg pMX/CD16 or pMX/CAR plasmid DNA using $CaCl_2$ precipitation (Invitrogen). The medium (10 mL) was replaced 6 h after transfection. The conditioned medium was collected 48 h after transfection, filtered through 0.45 μm pore-size filters, and kept at −80°C until use. The viral titer was determined by the transduction of Jurkat T cells (1×10^6 cells per well in 6-well plates) with serial dilutions of virus and analyzed for CD16 or CAR expression 4 days after infection. The retroviral supernatant titers were typically 1–5 $\times$ 10^5 IU (Infectious Units)/mL.

4.4. NK-92 Cell Line Transduction Using Retroviral Supernatant. The NK-92 cell line was resuspended in RPMI 1640 culture medium supplemented with 10% FBS and 100 IU/mL of recombinant IL-2, seeded at 1×10^6 cells in 1 mL per well into 6-well plates, and exposed to 2 $\times$ 2 mL of retroviral supernatant by spinoculation (2400 g, 1.5 h, and 32°C) in the presence of 4 μg/mL polybrene (Sigma, St. Quentin Fallavier, France). The culture medium was changed 24 h after infection. Mock (nontransduced) controls were performed in parallel, by which the supernatant of untransfected packaging cells was added to the NK-92 cell line. The transduction efficiencies were assessed 5 days later by flow cytometry after staining the CD16 and the CAR with a PE-conjugated mouse anti-CD16 (clone 3G8) or a PE-conjugated mouse anti-human IgG2a-Fc (clone HP6002), respectively.

4.5. Immunoselection of Transduced NK-92 Cell Lines. After transduction, NK-92^{CD16} and NK-92^{CAR} cells were stained with mouse anti-CD16 (clone 3G8) and anti-human IgG2a-Fc (clone HP6002), respectively, and immunoselected using anti-mouse-IgG coated beads (Dynabeads M-450, Dynal AS, Oslo, Norway), according to the supplier's instructions. Based on CD16 and CAR expression, the purity after immunoselection was >95%.

4.6. Cytotoxicity and ADCC Assay. Cytotoxic activity was assessed using a standard ^{51}Cr release assay. The target cells were labelled with 100 μCi ^{51}Cr for 1 h at 37°C, washed four times with culture medium, and plated at the indicated effector-to-target cell ratios in 96-well flat-bottom plates. The indicated mAb was incubated with the target cells for 20 mins at room temperature before the addition of effector cells. After 4 h incubation at 37°C, 25 μL of supernatant was removed from each well and mixed with 100 μL scintillation

fluid, and ^{51}Cr activity was counted in a scintillation counter (MicroBeta, Perkin Elmer, Courtaboeuf, France). Each test was performed in triplicate. The results are expressed as the percentage of lysis, which is calculated according to the following equation: (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100, where the experimental release represents mean counts per minute (cpm) of target cells in the presence of effector cells, spontaneous release represents the mean cpm of the target cells incubated without effector cells, and maximal release represents the mean cpm of the target cells incubated with 1% Triton X-100 (Sigma).

4.7. Mice. NOD/SCID-IL2R$\gamma^{-/-}$ (NSG-JAX) mice were bred and maintained under pathogen-free conditions at the Floralis Jean Roget Institute (UJF Grenoble, France) in sterile intraventilated cages. Mice were acclimated for 1 week before experimental use in Centre Léon Bérard animal facilities (Anican). All animal experiments were performed in compliance with French government guidelines and INSERM standards for experimental animal studies (agreement B69-388-0202). They were approved by the local ethics committee of Centre Léon Bérard, ENS, PBES, and P4 laboratory (CECAPP, Lyon, France). Body weight was monitored weekly and the mice were clinically examined throughout the study.

4.8. In Vivo Experimental Model. Six-week-old female NOD/SCID-IL2R$\gamma^{-/-}$ mice were injected subcutaneously in the left side with 5×10^6 BT474 cells. When tumors reached a minimal volume of 50 mm^3, mice were individually identified and randomly assigned to the control or treated groups (5 to 15 mice per group) and treatments were initiated. Next, tumor growth was monitored twice a week by measuring two perpendicular diameters with calipers. Tumor volume (V) was calculated using the following equation: $V = (a^2 \times b)/2$, where a is the width of the tumor (small diameter) and b the length (large diameter), both in millimeters. Nonirradiated NK-92^{NT}, NK-92^{CD16}, or NK-92^{CAR} effector cells (5×10^6 each) were injected with IP once a week for 4 weeks. Trastuzumab (15 mg/kg) or rituximab control antibody (15 mg/kg) was given IP 24 hours before injection of NK-92^{NT} or NK-92^{CD16}. Mice were sacrificed before the tumor volume reached 2500 mm^3. Each tumor was dissected and either fixed in formol and processed for histopathological examination or used for RNA extraction. Blood, liver, lung, and spleen from each animal were collected.

4.9. Immunohistochemistry. Frozen excision of BT474 tumors was analyzed by immunohistochemistry on 5 μm tissue sections using monoclonal anti-human CD45 (2D1, Dako) or Iba1 (019-19741, Wako) antibodies or direct CFSE visualization.

4.10. Coculture Experiment and IFN-γ Assay. Spleens from NSG mice were collected and separated in single-cell suspensions. An average of 1.5×10^6 splenocytes were obtained per spleen. Suspensions were stained with an antibody cocktail and then analyzed by flow cytometry for expression of CD11b (Mac-1) versus Gr-1 (Ly-6G) and CD11b versus CD16/32 (FcRγRIII/FcRγRII). Over 80% of splenocytes were positive for CD16/32 (see Figure 7, 1/3 experiments). NK-92 (NK-92^{NT}, NK-92^{CD16}, or NK-92^{CAR}) and splenocytes or the HER2 positive cell line BT474 were cocultured at an effector ratio of 5 : 1 and 2 : 1, respectively, in 96-well round bottom culture plates in RPMI 1640 supplemented with 10% FCS and IL2 (100 IU/mL). After 18 h, supernatants were collected and human IFN-γ content was estimated using an ELISA kit (Affymetrix, e-biosciences).

Abbreviations

CAR: Chimeric antigen receptor
FcγRIII: Fc receptor
IgG: Low affinity
FcεRIg: Fc receptor
IgE: High affinity
ADCC: Antibody-dependent cell-mediated cytotoxicity
NK: Natural killer
NSG: NOD scid gamma.

Conflict of Interests

The authors do not declare any competing financial interest.

Authors' Contribution

Béatrice Clémenceau and Sandrine Valsesia-Wittmann contributed equally to this work.

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Research Article

Regulation of Murine Ovarian Epithelial Carcinoma by Vaccination against the Cytoplasmic Domain of Anti-Müllerian Hormone Receptor II

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Anti-Müllerian hormone receptor, type II (AMHR2), is a differentiation protein expressed in 90% of primary epithelial ovarian carcinomas (EOCs), the most deadly gynecologic malignancy. We propose that AMHR2 may serve as a useful target for vaccination against EOC. To this end, we generated the recombinant 399-amino acid cytoplasmic domain of mouse AMHR2 (AMHR2-CD) and tested its efficacy as a vaccine target in inhibiting growth of the ID8 transplantable EOC cell line in C57BL/6 mice and in preventing growth of autochthonous EOCs that occur spontaneously in transgenic mice. We found that AMHR2-CD immunization of C57BL/6 females induced a prominent antigen-specific proinflammatory CD4+ T cell response that resulted in a mild transient autoimmune oophoritis that resolved rapidly with no detectable lingering adverse effects on ovarian function. AMHR2-CD vaccination significantly inhibited ID8 tumor growth when administered either prophylactically or therapeutically, and protection against EOC growth was passively transferred into naive recipients with AMHR2-CD-primed CD4+ T cells but not with primed B cells. In addition, prophylactic AMHR2-CD vaccination of TgMISIIR-TAg transgenic mice significantly inhibited growth of autochthonous EOCs and provided a 41.7% increase in mean overall survival. We conclude that AMHR2-CD vaccination provides effective immunotherapy of EOC with relatively benign autoimmune complications.

1. Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecologic malignancies in the United States [1, 2]. Approximately 60% of ovarian cancers are diagnosed at late stages, and although initial responses to the current standard of care are high, most patients have disease recurrence resulting in a five-year overall survival (OS) rate slightly over 45% [2, 3]. The high rate of ovarian cancer recurrence and the low five-year survival rate indicate the urgency for more effective ways to control this disease.

Induction of ovarian tumor immunity through vaccination is a promising approach and finds support from the increased OS observed in patients whose ovarian tumors

are infiltrated by T cells [4]. Several therapeutic ovarian cancer vaccine strategies have been employed using whole tumor homogenate strategies as well as approaches involving targeted immunity against tumor associated antigens (TAA) overexpressed in ovarian malignancies including human epidermal growth factor receptor 2 (HER2), cancer-testis antigen 1 (CTAG1B or NY-ESO-1), or cancer antigen 25 (CA-125) [5]. Thus far, targeted immunity against these non-ovarian-specific TAA has provided modest therapeutic results [6–8].

In contrast, vaccination against tissue-specific differentiation antigens has not been fully exploited for providing ovarian cancer therapy despite the ability of such targeted vaccinations to increase OS in melanoma and prostate cancer patients [9–11]. Thus, vaccination against differentiation proteins expressed at immunogenic levels predominantly in the tissue from which the tumor is derived may provide effective immunotherapy against established tumors and at the same time substantially lower risk of inducing systemic autoimmune inflammatory complications.

We selected mouse anti-Müllerian hormone receptor II (AMHR2, GenBank ID: 110542) as our target differentiation protein for ovarian cancer vaccination because its full-length expression in normal human tissues is confined to the ovary and because it is also expressed in the vast majority of human EOCs including 90% of primary EOCs, 78% of borderline malignancies, 77–86% of non-EOC ovarian tumors, and 56% of malignant ascites from grades III-IV ovarian cancers [12–14].

AMHR2 is a serine/threonine kinase receptor homologous to type II receptors of the transforming growth factor beta (TGFβ) family [15]. The human *AMHR2* gene contains 11 exons with seven known alternatively spliced variants producing three known coded proteins, one additional variant with protein coding features, and three noncoding transcripts with no open reading frames [16, 17]. In adult women, the longest human protein coding transcript for a 573-amino acid long protein is normally expressed only in the ovary and comprises a 127-amino acid extracellular domain, a 26-amino acid transmembrane domain, and a 403-amino acid cytoplasmic domain [16, 17]. AMHR2 signaling causes regression of the Müllerian ducts during male development and regulates oocyte development and follicle production in adult females thereby providing substantial control of ovarian reserve and fertility [15, 18–20].

Based on its expression in 90% of primary human EOCs as well as on its relatively confined distribution in normal human tissues, we hypothesized that AMHR2 vaccination would provide effective immunotherapy against EOC without producing extensive autoimmune complications. We tested our hypothesis using both transplantable and autochthonous mouse models for EOC. Mouse ID8 cells, derived from repeated *in vitro* passage of mouse ovarian surface epithelial cells (MOSEC), form EOCs when inoculated into C57BL/6 mice [21]. TgMISIIR-TAg transgenic mice develop bilateral autochthonous EOCs due to expression of the simian virus 40 large T antigen (SV40-TAg) under control of the *AMHR2* promoter [22].

All efforts to generate a full-length AMHR2 protein proved futile due to extensive toxicity in all expression systems tested. We resolved this toxicity problem by generating a recombinant mouse AMHR2 protein consisting of a 399-amino acid sequence of the cytoplasmic domain (AMHR2-CD) and found that immunization with this fragment resulted in a prominent proinflammatory T cell response accompanied by extremely high IgG antibody titers. Vaccination with AMHR2-CD provided highly significant T cell-mediated prophylaxis and therapy against ID8 EOC and mediated significant prophylaxis against the development of autochthonous EOCs in TgMISIIR-TAg transgenic mice. Moreover, the protection against tumor growth was accompanied by a rather benign autoimmune phenotype. Our data indicate that targeted vaccination against AMHR2-CD provides relatively safe and highly effective therapy against EOC.

2. Materials and Methods

2.1. Generation of Recombinant Mouse AMHR2-CD. mRNA was extracted from ovaries of 8-week-old female C57BL/6 mice. Primer pairs designed to amplify the AMHR2 sequence 170–568 were used to generate the entire 399-amino acid cytoplasmic domain of mouse AMHR2 by RT-PCR [23]. To optimize protein folding and enhance overall yield, substitutions for native codon sequences were made (Dapcel, Cleveland, OH), and the optimized cDNA was inserted into the NdeI-Bam HI site of pET-3a (Novagen, Darmstadt, Germany) thereby providing a C-terminal 6xHis-tagged recombinant protein (Figure 1(a)). Plasmids containing these inserts were transformed in *E. coli* (Lucigen, Middleton, WI). High level expression colonies were selected following induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG, Amresco, Solon, OH) and were sequenced for confirming proper orientation and alignment. The 6xHis-tagged AMHR2-CD was purified under denaturing conditions using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen Sciences, Germantown, MD). The purified AMHR2-CD was electrophoresed on denaturing SDS-PAGE gels (Bio-Rad, Hercules, CA) and blotted onto immunoblot PVDF membrane (Bio-Rad). Immune detection of AMHR2-CD was performed using the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ) with HRP-conjugated His antibody (Qiagen). Prior to use, the 6xHis-tagged AMHR2-CD was purified by reverse phase HPLC to yield endotoxin-free protein [24]. Levels of endotoxin were <0.05 endotoxin units (<5 pg) per mg of recombinant protein.

2.2. Mice and Immunization. Female C57BL/6 mice served as recipients of ID8 tumors. They were obtained commercially (Jackson Laboratory, Bar Harbor, ME) at six weeks of age and immunized at 7–10 weeks of age by subcutaneous injection in the abdominal flanks with 100 μg of recombinant mouse AMHR2-CD in 200 μL of an emulsion of equal volumes of water and complete Freund's adjuvant (CFA, Difco, Detroit, MI) containing 400 μg of *Mycobacteria tuberculosis*. TgMlSIIR-TAg (DR26 line) transgenic mice (provided by DDC) were maintained by breeding male TgMISIIR-TAg

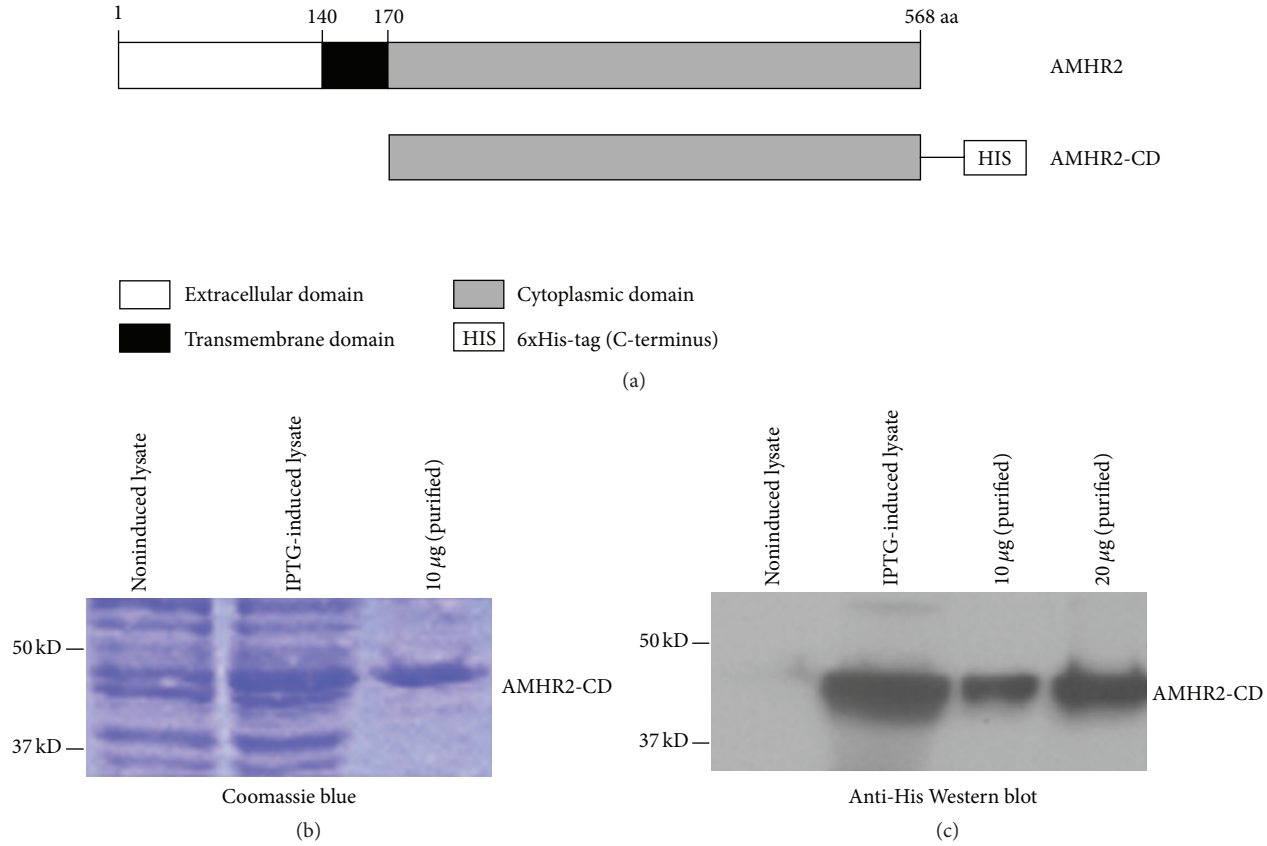

Figure 1: *Generation of recombinant mouse AMHR2-CD*. (a) Schematic representation of full-length AMHR2 showing the extracellular, transmembrane, and cytoplasmic domains with a C-terminal 6xHis-tagged AMHR2-CD variant. (b) Expression of AMHR2-CD in noninduced, IPTG-induced, and Ni-NTA affinity purified AMHR2-CD shown on an SDS-PAGE gel stained with Coomassie blue. (c) Anti-His Western blot of SDS-PAGE gel showing expression of AMHR2-CD in noninduced, IPTG-induced, and two doses of Ni-NTA affinity purified AMHR2-CD.

(H-2^b) mice to wild-type syngeneic C57BL/6 females (Jackson Laboratory). TgMISIIR-TAg mice were immunized at 6-7 weeks of age with 100 μg of recombinant mouse AMHR2-CD in CFA as described above. To determine fertility phenotypes, age-matched test and control vaccinated C57BL/6 female mice were mated with the same C57BL/6 males. All protocols were preapproved by Cleveland Clinic's Institutional Animal Care and Use Committee.

2.3. Tumor Inoculation and Measurement. The ID8 EOC cell line was generously provided by Dr. Kathy Roby (University of Kansas Medical Center, Kansas City, KS). ID8 cells were cultured in 75 or 225 cm^2 tissue culture flasks (BD Biosciences, Franklin Lakes, NJ) in DMEM (Mediatech Cellgro, Manassas, VA) containing 4% fetal bovine serum (Thermo Scientific Hyclone, Logan, UT), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA), and insulin-transferrin-sodium selenite media supplement (Sigma-Aldrich, St. Louis, MO) until the cells became 70–80 % confluent. Cells were harvested by trypsinization and washed twice with PBS. Female C57BL/6 mice were inoculated subcutaneously in the left dorsal flank with 5×10^6 ID8 cells. Growth of ID8 tumors was assessed regularly by using a Vernier caliper to measure length × width. Tumor growth endpoint was determined by a measurement in any direction of 17 mm.

2.4. In Vivo Imaging and Measurement of Autochthonous Ovarian Tumors. Bilateral ovarian tumor growth in female transgenic mice was measured monthly by ultrasound using the Vevo 770 high-resolution *in vivo* microimaging system for small animals (VisualSonics, Toronto, Canada). Real-time imaging of the abdomen was performed using the RMV704 low frequency probe/scan head and aqueous conductive gel after removing hair from the abdominal region. Anesthesia for immobilization was administered using a nose cone with continuous flow of 1-2% vol/vol isoflurane during the image acquisition period lasting less than 30 minutes, and oxygen supply was continuously maintained. The probe/scan head was moved over the abdominal area very gently after applying aqueous conductive gel. Measurements and calculation of tumor area were performed using the Vevo software B-Mode measurement tool allowing for a 2D assessment of ovarian tumor size *in vivo* with the polygon region of interest setting (VisualSonics). Measurement of solid tumor size by B-mode sonography has been shown to correlate well with histopathologic measurement [25].

2.5. RT-PCR. Tissues were excised and stored frozen in RNA-Later (Life Technologies, Grand Island, NY). RNA was extracted by tissue homogenization in TRIZOL reagent (Invitrogen), and cDNA was generated from bulk RNA using

Table 1: Primer pairs used for cloning, qRT-PCR, detection of transgene, and conventional RT-PCR.

Protein	Sequence (5′-3′)	Amplicon length (bp)
Cloning of AMHR2-CD		
AMHR2-CD		
Forward	GGATCCAAGGCCTGCAGAGTGCAAGGTG	1209
Reverse	AAGCTTCTACTCATTTACATACACCTG	
TgMISIIR-TAg transgene expression		
SV40-TAg		
Forward	TGCATGGTGTACAACATTCC	773
Reverse	TTGGGACTGTGAATCAATGCC	
qRT-PCR		
IFNγ		
Forward	GGATATCTGGAGGAACTGGCAA	110
Reverse	TGATGGCCTGATTGTCTTTCAA	
TNFα		
Forward	CGAGTGACAAGCCTGTAGCC	209
Reverse	GTGGGTGAGGAGCACGTAGT	
IL-2		
Forward	GCAGGCCACAGAATTGAAAG	207
Reverse	TCCACCACAGTTGCTGACTC	
CD4		
Forward	ACACACCTGTGCAAGAAGCA	69
Reverse	GCTCTTGTTGGTTGGGAATC	
CD8		
Forward	TTACATCTGGGCACCCTTG	132
Reverse	TTGCCTTCCTGTCTGACTAGC	
NKR-P1A		
Forward	GGCTTGGCATGAGTCACC	75
Reverse	TTCAGAGCCAACCTGTGTGA	
β-actin		
Forward	GGTCATCACTATTGGCAACG	133
Reverse	ACGGATGTCAACGTCACACT	
Conventional RT-PCR		
AMHR2		
Forward	GTATCCGCTGCCTCTACAGC	193
Reverse	CAGAAGTCAGTGCCACAGGA	
β-actin		
Forward	GGTCATCACTATTGGCAACG	133
Reverse	ACGGATGTCAACGTCACACT	

Superscript III (Invitrogen). Gene expression was quantified by qRT-PCR using SYBR Green PCR mix (Applied Biosystems, Carlsbad, CA) with gene-specific primers (Table 1). Relative gene expression was assessed by normalization of each test gene expression level to β-actin expression levels in each individual tissue. Gene expression was determined by conventional RT-PCR using AMHR2-specific and β-actin-specific primers (Table 1). After amplification through 30 cycles, PCR products were separated on agarose gels (2% in 1 TBE buffer) and visualized under ultraviolet light after staining with ethidium bromide. Transgene expression in offspring of TgMISIIR-TAg mice was determined by PCR amplification of a 773 bp fragment of SV40-TAg using primer pairs as previously described [22] (Table 1).

2.6. Flow Cytometry Analysis of Tumor Infiltrating Lymphocytes (TILs). TILs were isolated from ID8 tumors by digestion of minced tumor for 30 minutes at 37°C in HBSS containing 50 KU of DNase I (Sigma-Aldrich) and 0.2 mg/mL collagenase II (Life Technologies) followed by discontinuous gradient centrifugation. The partially purified TILs were treated with Fcγ III/II receptor antibody (BD Biosciences) in PBS containing 0.5% BSA and 0.05% sodium azide and double-stained with FITC-conjugated anti-mouse CD3 and either PE-conjugated anti-mouse CD4 or PE-conjugated anti-mouse CD8 (BD Biosciences). The CD3+ T cell population was gated and analyzed for percentages of CD4+ and CD8+ T cells. Data collected on 30,000 total events were analyzed using FlowJo software (BD Biosciences).

2.7. Passive Transfer of Tumor Immunity. Ten days after immunization of female C57BL/6 mice with AMHR2-CD or ovalbumin (OVA, Sigma-Aldrich) as an irrelevant control immunogen, LNCs at 5×10^6 cells/mL were activated *in vitro* with 20 μg/mL of immunogen in the presence of IL-12 (10 ng/mL) and IL-18 (10 ng/mL; Peprotech, Rocky Hill, NJ) in 24-well flat-bottom Falcon plates (BD Biosciences) in a total volume of 2.0 mL/well in DMEM supplemented as described above. After 3 days of restimulation, 2×10^7 activated whole LNCs were injected intraperitoneally into sublethally.

γ-irradiated (5 Gy) naive female recipients. In another protocol, C57BL/6 female mice were immunized with either AMHR2-CD or OVA, and four weeks later, three groups of cells were injected intraperitoneally into sublethally γ-irradiated (5 Gy) naive female recipients including 7.5×10^7 whole splenocytes reactivated with immunogen, IL-12, and IL-18 as described above, 2×10^7 similarly reactivated CD4+ T cells purified from whole splenocytes by magnetic bead separation, and 2×10^7 nonreactivated B220+ B cells also purified from whole splenocytes by magnetic bead separation. In all cases, hosts were inoculated subcutaneously on the day after cell transfer with 5×10^6 ID8 cells, and tumor growth was assessed regularly as described above. Purities of enriched cells were determined by flow cytometry analysis using CellQuest software (BD Biosciences) and were consistently found to be >90%.

2.8. Immunologic Assays. T cell proliferation, ELISA assays for cytokine production, and immunohistochemical analysis were performed as previously described [26] and are detailed in supplemental material.

2.9. Biostatistical Analysis. Differences between mRNA expression levels and mean tumor weights were compared using Student's *t*-test. Differences between tumor growth curves were compared by unweighted one-way ANOVA, and differences in mouse survival curves were compared using the log-rank test.

3. Results

3.1. Generation of Recombinant Mouse AMHR2-CD. All attempts to express the full-length sequence of mouse AMHR2 in any expression system consistently caused cytotoxicity and failure to produce high expression colonies. To overcome this persistent cytotoxic effect, we expressed the longest hydrophilic domain of mouse AMHR2 consisting of the 170–568 sequence comprising the 399 amino acids of the entire cytoplasmic domain (Figure 1(a)). The Ni-NTA affinity purified C-terminal 6xHis-tagged protein migrated as a ~44 kD protein as determined by Coomassie blue staining of an SDS-PAGE gel (Figure 1(b)) and by Western blot immunostaining using HRP-conjugated His-specific antibody (Figure 1(c)).

3.2. Immunogenicity of AMHR2-CD. Ten days after AMHR2-CD immunization of female C57BL/6 mice, LNC showed proliferation in a dose response manner to AMHR2-CD but not to recombinant human cochlin, a control protein generated and purified in a manner similar to AMHR2-CD (Figure 2(a)) [27]. This antigen-specific proliferation by LNC was elicited from purified CD4+ T cells but not from purified CD8+ T cells (Figure 2(b)) and was inhibited by treatment of cultures with CD4-specific but not CD8-specific antibodies (Figure 2(c)). Four weeks after immunization, ELISA analysis of supernatants from immunogen-stimulated splenocytes showed a predominant proinflammatory response to AMHR2-CD with high production of interferon gamma (IFNγ) and with relatively low production of IL-2, IL-4, and IL-5 (Figure 2(d)). Purification of T cell subsets from the whole splenocyte population showed that CD4+ but not CD8+ T cells produced the IFNγ in response to AMHR2-CD (Figure 2(e)). Two months after immunization, serum levels of AMHR2-CD-specific IgG were detectable even at titers exceeding 1 : 50,000 dilution (Figure 2(f)).

3.3. Benign Transient Ovarian Inflammation following AMHR2-CD Immunization. We next examined the potential of AMHR2-CD immunization to induce ovarian autoimmunity. Four and eight weeks after AMHR2-CD immunization of C57BL/6 female mice, ovarian IFNγ gene expression was measured by qRT-PCR. Relative ovarian IFNγ gene expression was modestly elevated 4 weeks after AMHR2-CD immunization but not after immunization with CFA alone (Figure 3(a)). Eight weeks after immunization, relative ovarian IFNγ gene expression was similar in both immunized groups of mice. Most notably, the transiently elevated IFNγ gene expression observed in AMHR2-CD immunized mice at 4 weeks was only 3-fold higher than CFA control mice, far lower than what we had previously observed in lactating breast tissues from mice immunized with α-lactalbumin where the levels of IFNγ gene expression were more than 50 times greater than those occurring in CFA immunized control mice and were associated with substantial breast inflammation and lactation failure [26]. Despite repeated attempts to detect CD3+ T cells in ovaries by immunohistochemical analysis at 4, 8, and 12 weeks after AMHR2-CD immunization, we could not find any infiltrates. More importantly, the low level transient expression of IFNγ in ovaries of AMHR2-CD immunized mice was not associated with any detectable effect on ovarian function determined by fertility over four sequential mating cycles during which no significant differences ($P > 0.60$) occurred in the number of pups generated per litter between AMHR2-CD and CFA immunized mice (Figure 3(b)). Moreover, it remains highly unlikely that AMHR2-CD immunization induces any substantial nonovarian autoimmune inflammation since we found that AMHR2 gene expression was readily detected in the ovaries and ID8 ovarian tumor cells and was not detected at any appreciable levels in normal mouse uterus, stomach, spleen, heart, lung, kidney, and liver (Figure 3(c)).

3.4. Inhibition of Tumor Growth in Mice Immunized with AMHR2-CD. We next determined whether vaccination with AMHR2-CD would inhibit growth of transplantable ID8 tumors in C57BL/6 female mice. We found that ID8 tumor

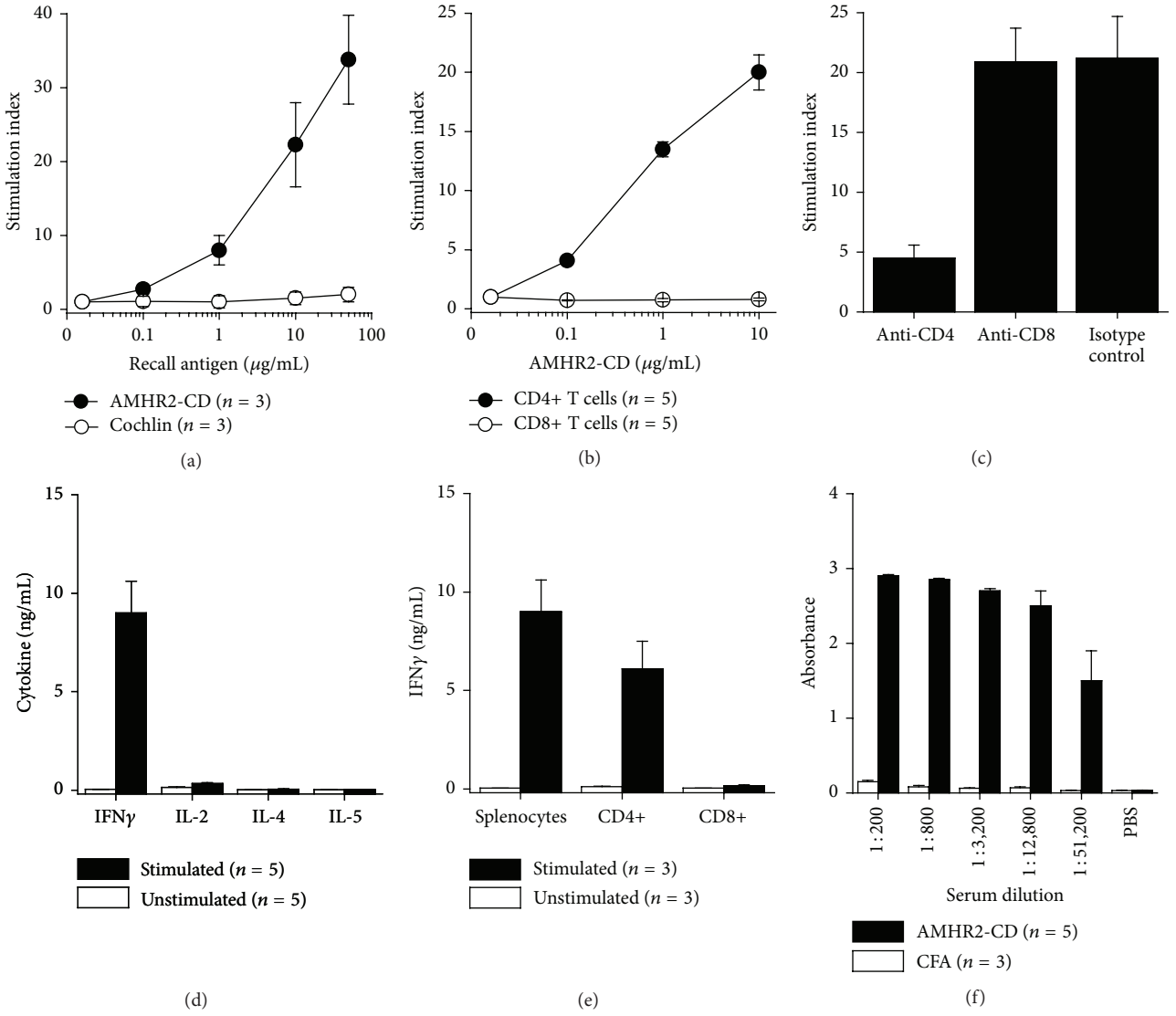

Figure 2: *Immunogenicity of AMHR2-CD*. Female C57BL/6 mice were immunized with AMHR2-CD in CFA, and LNC or splenocytes were cultured *in vitro* for assessment of proliferation and cytokine production. (a) Ten-day primed LNC showed marked antigen-specific recall proliferative responses to AMHR2-CD over several logs of antigen concentration. (b) The response to AMHR2-CD was elicited by CD4+ T cells but not by CD8+ T cells purified by magnetic bead separation. (c) Proliferative responses to AMHR2-CD were markedly inhibited in the presence of CD4 antibody but not in the presence of CD8 or isotype control antibodies. (d) Four weeks after immunization, splenocytes were reactivated with immunogen and ELISA analysis of 72-hour culture supernatants showed that recall responses to AMHR2-CD involved a proinflammatory phenotype with elevated production of IFNγ and minimal production of IL-2, IL-4, and IL-5. (e) Splenocyte production of IFNγ was elicited from purified CD4+ T cells but not from purified CD8+ T cells. (f) Two months after immunization, serum levels of AMHR2-CD-specific IgG were detectable even at titers over 1 : 50,000 dilution. PBS was substituted for diluted sera in the PBS control. Error bars show ±SD.

growth was inhibited in mice prophylactically vaccinated 15 days (Figure 4(a), $P < 0.001$), 7 days (Figure 4(b), $P < 0.001$), or 1 day (Figure 4(c), $P < 0.05$) prior to inoculation of ID8 ovarian tumor cells. In addition, AMHR2-CD vaccination resulted in a significantly decreased overall tumor load as measured by final ID8 tumor weight at termination of experiments in mice vaccinated 7 days ($P < 0.01$) and 1 day ($P < 0.05$) prior to ID8 inoculation (Figure 4(d)). AMHR2-CD vaccination was also effective as therapy against EOC. Vaccination with AMHR2-CD 60 days after inoculation of ID8 tumors significantly inhibited the growth of established, palpable ID8 tumors ($P < 0.05$, Figure 4(e)). We also found that vaccination with AMHR2-CD significantly inhibited the growth of autochthonous EOCs that develop spontaneously in TgMlSIIR-TAg transgenic mice ($P < 0.0001$, Figure 4(f)). Moreover, this inhibition in tumor growth was accompanied by a highly significant increased OS when compared to CFA vaccinated control mice ($P < 0.0005$, Figure 4(g)). This enhanced lifespan in AMHR2-CD vaccinated mice (mean 191.25 days ±22.95) compared to CFA vaccinated control mice (mean 135 days ±13.89) represents a dramatic 41.7% increase in OS.

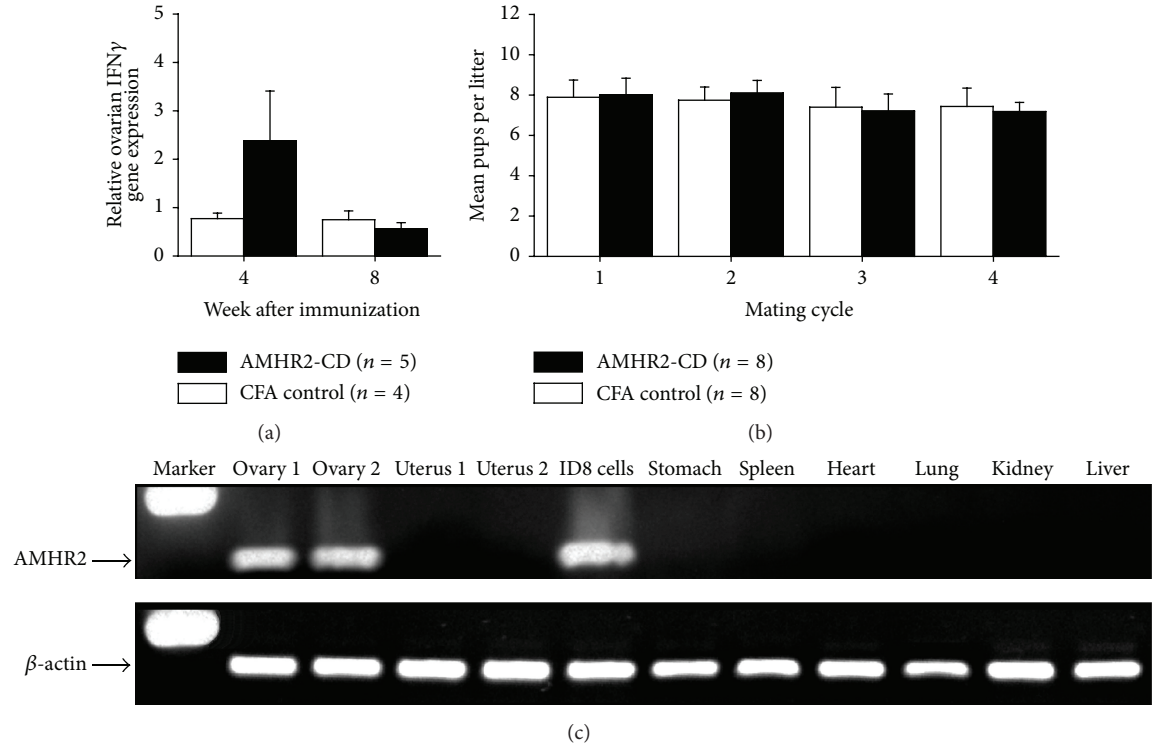

Figure 3: *Benign transient ovarian inflammation following AMHR2-CD immunization.* (a) Relative ovarian IFNγ gene expression was elevated 4 weeks after immunization with AMHR2-CD but not after immunization with CFA alone. At eight weeks after immunization, relative ovarian IFNγ gene expression was similar in both immunized groups of mice. (b) The low level transient expression of IFNγ in ovaries of AMHR2-CD immunized mice was not associated with any detectable effect on ovarian function as determined by assessing fertility defined by pup production over four sequential mating cycles in female C57BL/6 mice immunized with AMHR2-CD and control mice immunized with CFA alone. (c) AMHR2 gene expression was confined to ovaries and ID8 ovarian tumor cells and was not detected in normal uterus, stomach, spleen, heart, lung, kidney, and liver. Error bars show ±SD.

3.5. ID8 Tumor Analysis. At the termination of experiments, tumors were analyzed for inflammatory infiltrates. Immunohistochemical analysis consistently showed extensive infiltration of CD3+ T cells in tumors from AMHR2-CD vaccinated mice (Figure 5(a)). We found no infiltrates in tumors from mice immunized with CFA alone (data not shown). Flow cytometry analysis of TILs showed a pronounced increase of CD4+ T cells in tumors from mice vaccinated with AMHR2-CD compared to control mice immunized with CFA alone (40.7% versus 11.7%, Figure 5(b)). Substantial increases of CD8+ T cells in tumors did not occur in AMHR2-CD immunized mice compared to CFA immunized control mice (10.5% versus 7.4%, resp.). We next analyzed tumor RNA for gene expression of proinflammatory factors by qRT-PCR. When compared to tumors from CFA immunized control mice, tumors from AMHR2-CD immunized mice consistently showed significantly increased relative gene expression ($P < 0.05$ in all cases) for CD4, IFNγ, tumor necrosis factor alpha (TNFα), IL-2, and the natural killer cell receptor NKR-P1A [28] but not for CD8 (Figure 5(c)). These data indicate the induction of a proinflammatory immune milieu within the ID8 tumor following immunization with AMHR2-CD.

3.6. Passive Transfer of Tumor Immunity with CD4+ T Cells. All recipient mice were inoculated with ID8 tumor cells on the day after cell transfer. Tumor growth was significantly inhibited in mice transferred with AMHR2-CD-specific LNCs ($P = 0.04$, Figure 6(a)) and splenocytes ($P < 0.01$, Figure 6(b)) when compared to mice receiving OVA-specific LNCs. At 190 days after transfer of primed splenocytes and tumor inoculation, mean tumor weights were significantly lower in recipients of AMHR2-CD-specific splenocytes compared to recipients of OVA-specific splenocytes ($P < 0.05$, Figure 6(c)). Transfer of AMHR2-CD-specific CD4+ T cells purified from 4-week primed splenocytes resulted in significant inhibition of ID8 tumor growth compared to transfer of purified OVA-specific CD4+ T cells ($P < 0.0004$, Figure 6(d)) whereas transfer of AMHR2-CD-primed B220+ B cells purified from 4-week primed splenocytes did not significantly inhibit ID8 tumor growth compared to transfer of OVA-primed B220+ B cells ($P = 0.07$, Figure 6(d)). Thus, AMHR2-CD-specific proinflammatory CD4+ T cells are sufficient for transferring immune protection against the growth of EOC.

4. Discussion

Our data derived from both transplantable and autochthonous ovarian tumor models show that vaccination against AMHR2-CD, a defined fragment of an ovarian differentiation

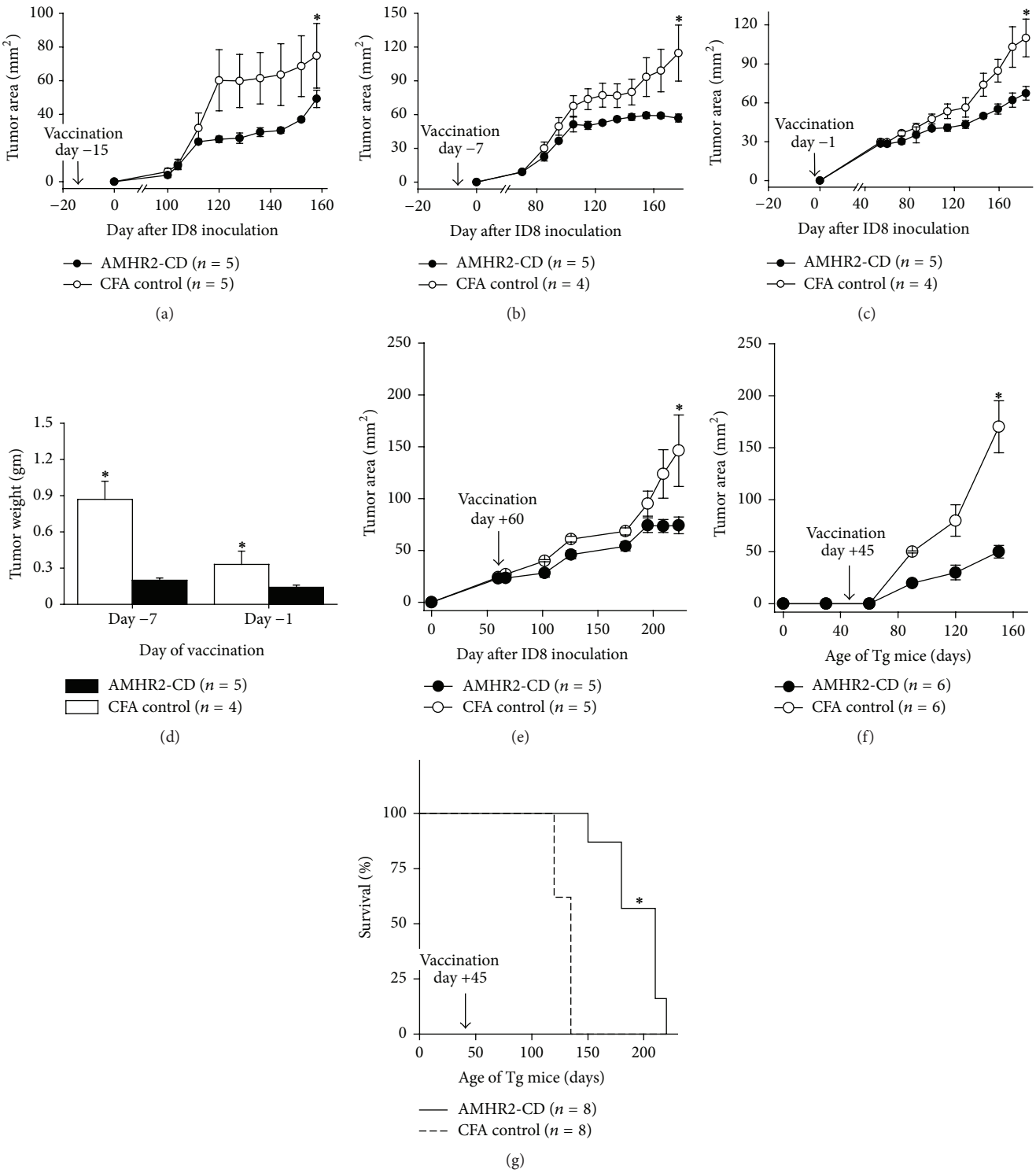

Figure 4: *Inhibition of tumor growth in mice immunized with AMHR2-CD*. ID8 tumor growth was inhibited in mice prophylactically vaccinated (a) 15 days, (b) 7 days, or (c) 1 day prior to inoculation of tumor cells. (d) AMHR2-CD vaccination resulted in a significantly decreased overall tumor load as measured by final tumor weight at termination of experiments in mice vaccinated 7 days and 1 day prior to ID8 inoculation. (e) Therapeutic vaccination with AMHR2-CD 60 days after inoculation of ID8 tumors significantly inhibited the growth of established, palpable, and growing ID8 tumors. (f) Prophylactic vaccination of female TgMlSIIR-TAg transgenic mice at 6-7 weeks of age with AMHR2-CD resulted in a highly significant inhibition in growth of autochthonous EOC. (g) Prophylactic AMHR2-CD vaccination of female TgMlSIIR-TAg transgenic mice at 6-7 weeks of age resulted in a highly significant 41.7% mean increased OS compared to control mice vaccinated with CFA alone. Asterisks indicate statistical significance. Error bars show ±SD.

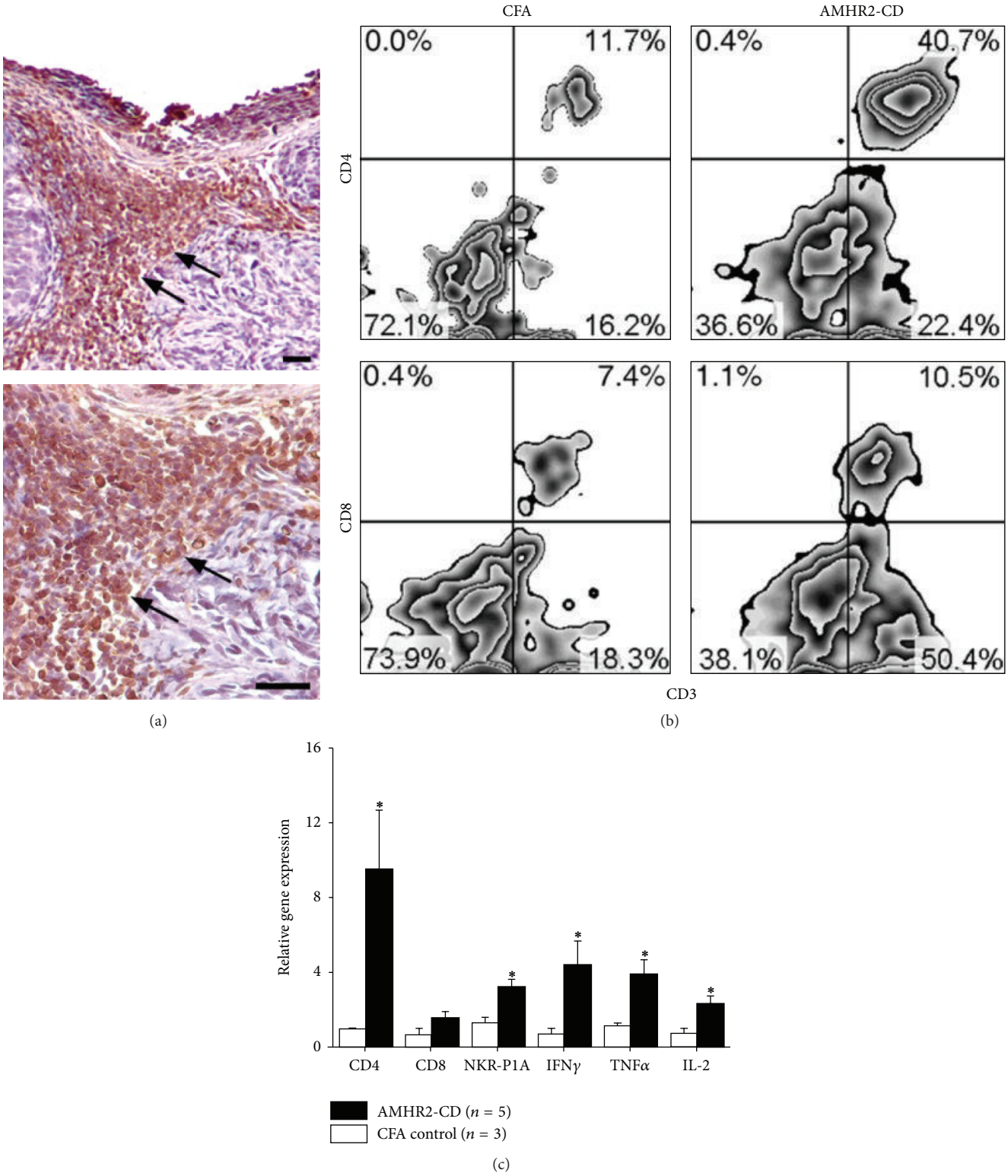

Figure 5: *Tumor analysis*. (a) Arrows show extensive infiltration of CD3+ T cells in an ID8 tumor from AMHR2-CD vaccinated mice in lower resolution (upper panel) and higher resolution (lower panel) images. Inflammatory infiltrates of CD3+ T cells were never observed in control mice vaccinated with CFA alone. (b) Flow cytometry analysis of TILs gated on the CD3+ T cell population showed a pronounced increase in percentages of CD4+ T cells but not CD8+ T cells in tumor infiltrates from mice vaccinated with AMHR2-CD compared to control mice immunized with CFA alone. Data shown are representative of three experiments yielding similar results. (c) Tumors from AMHR2-CD immunized mice consistently showed increased relative gene expression for CD4, IFNγ, TNFα, NKR-P1A, and IL-2 but not for CD8. Asterisks indicate statistical significance. Error bars show ±SD.

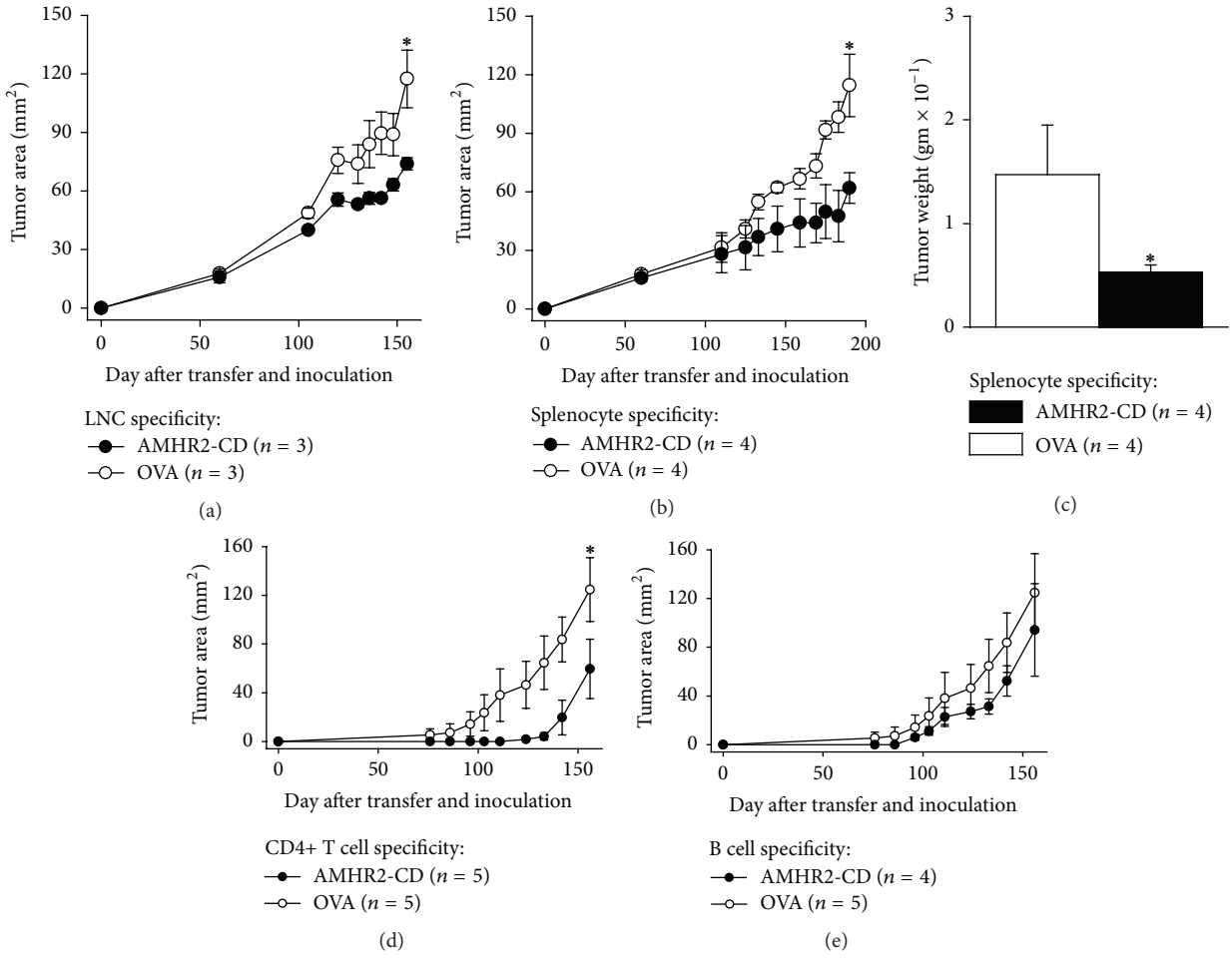

Figure 6: *Passive transfer of immune protection against tumor growth with CD4+ T cells*. Recipient mice were inoculated with ID8 tumor cells on the day after cell transfer. Growth of ID8 tumors was inhibited in mice transferred with AMHR2-CD-specific (a) LNCs and (b) splenocytes. (c) At 190 days after splenocyte transfer and inoculation, mean tumor weights were lower in recipients of AMHR2-CD-specific splenocytes compared to recipients of OVA-specific splenocytes. Transfer of purified AMHR2-CD-specific (d) CD4+ T cells but not (e) B220+ B cells inhibited ID8 tumor growth. Asterisks indicate statistical significance. Error bars show ±SE.

protein expressed in the vast majority of human EOCs, provides effective therapy and prophylaxis against ovarian cancer. It is particularly encouraging that the inhibition of tumor growth was accompanied by a mild ovarian inflammation that resolved quickly with no detectable effects on fertility over the course of several subsequent mating cycles. This rather benign autoimmune phenotype was associated with a significant inhibition of tumor growth when vaccination occurred as a therapeutic intervention. It is important to note that the appearance of the therapeutic effect took over five months to clearly manifest as defined by a complete separation of the tumor growth curves (Figure 4(e)), thereby implying that earlier vaccination as a preventive strategy would be even more effective in controlling EOC. Indeed, the highly significant 41.7% increased OS that occurred when TgMISIIR-TAg mice were vaccinated prophylactically supports this view. However, in light of several reports indicating extraovarian gene and protein expression of AMHR2, the likelihood of using AMHR2-CD vaccination as prophylaxis against EOC seems unlikely.

AMHR2 gene and protein expression have been repeatedly detected in adult motor neurons in mice [29–32], in normal adult rat endometrium, at low levels in the normal rat uterus, and at substantially higher levels in the gravid rat uterus [33]. Although *AMHR2* gene expression has also been shown to occur in brain, adrenal, and lung tissues of adult male mice, the detected levels were less than 1% of those occurring in adult testes, and protein detection was either not reported or not prominent [34]. Similarly, *AMHR2* gene expression has been detected in normal rat and normal human breast tissues, but detection of the AMHR2 protein was not reported in either of these tissues [35].

Despite extensive literature on extraovarian gene expression of *AMHR2*, recent rigorous studies have provided more precise understanding of the subtle but important features involved in *AMHR2* gene expression in different normal human tissues. Quantitative estimates of transcript abundance by mRNA sequencing have shown that expression of the full-length AMHR2 transcript is confined to the ovary in adult women whereas alternative splice isoforms

coding for known truncated AMHR2 proteins as well as several noncoding transcripts are expressed in the cortex and medulla of the adrenal gland and at substantially lower levels in the spleen and exocrine cells of the pancreas [17]. Expression of truncated isoforms of AMHR2 also occurs in human skeletal muscle and heart but at levels, respectively, representing only 4.2% and 1.5% of the level expressed in the ovary. Thus, it seems that the extraovarian expression of AMHR2 reported in the literature refers to alternatively spliced transcripts that either code for truncated variant AMHR2 proteins or represent noncoding transcripts with no open reading frames [16, 17]. Noncoding AMHR2 transcripts have been shown to play a role in regulating AMHR2-mediated signaling [34] whereas all of the truncated AMHR2 transcripts with open reading frames have substantial deletions in the cytoplasmic domain of AMHR2 and as such are not capable of translating the complete AMHR2-CD sequence [16, 17]. Thus, the substantial AMHR2 deletions in nonovarian tissues may preclude the development of any life-threatening peripheral autoimmunity as evidenced by the lack of any observed extraovarian autoimmunity in our AMHR2-CD vaccinated mice and by the dramatically increased OS occurring in female TgMISIIR-TAg transgenic mice vaccinated prophylactically against AMHR2-CD.

The lack of any observed nonovarian autoimmunity in females provides several noteworthy considerations when selecting cancer vaccine targets including the importance of recognizing differences between immunogenic and non-immunogenic tissue expression levels when anticipating autoimmune consequences of cancer vaccination. In addition, one must evaluate the significance of species-specific differences in tissue expression of cancer vaccine targets, since, unlike the mouse [35], AMHR2 transcripts have not been detected in any of the normal human brain tissues examined [17]. In any event, extraovarian expression of AMHR2 transcripts does little to diminish the usefulness of AMHR2-CD as an immune target for immunotherapy of EOC particularly in light of the urgent need to improve the poor prognosis of women diagnosed with EOC and the unusually high immunogenicity of AMHR2-CD indicated by T cell production of high levels of IFNγ and induction of extremely high serum antibody titers with prominent detection of AMHR2-CD-specific IgG occurring even at serum dilutions exceeding 1 : 50,000 (Figure 2(f)).

It is notable that a single immunization with AMHR2-CD is capable of inducing sufficient tumor immunity without eliciting a detectable CD8 T cell response. Although immunization with tissue-specific self-proteins often fails to elicit CD8 T cell responses, it may be presumptuous to conclude that such failure is due to the single immunization protocol. Indeed, we have previously shown that CD4 and CD8 T cell responses occur following single immunizations with α-lactalbumin and uroplakin II for effective induction of autoimmune breast failure and interstitial cystitis, respectively [26, 36]. In fact, booster immunizations often diminish effective immunity [37]. Thus, failure to induce CD8 T cell responses cannot be explained simply by inadequate priming and may instead be due to the unavailability of autoreactive CD8 T cells capable of responding to a specific self-protein possibly as a result of a more efficient thymic deletion of the high affinity CD8 T cell repertoire. In such cases, booster immunizations would simply recruit low affinity T cell clones representing a nondominant or cryptic T cell repertoire capable of limited clinical impact. Although optimal tumoricidal activity may typically occur when tumor responses involve both CD4 and CD8 T cells [38], CD4 T cells by themselves can provide powerful tumor immunity often exceeding that provided by CD8 T cells even when tumors fail to express major histocompatibility complex (MHC) class II molecules [39]. Studies to determine the basis for the unusually high immunogenicity of AMHR2-CD are ongoing.

A variety of mechanisms may contribute to CD4-mediated tumor immunity including induction of help for tumor responsive CD8+ T cell responses, induction of tumor cytotoxicity, upregulation of expression of MHC molecules for enhancing recognition of tumor antigens, inhibition of angiogenesis, and induction of tumor dormancy (reviewed in [40]). Most of these mechanisms are directly or indirectly related to upregulation of IFNγ and TNFα gene expression both of which occurred in tumors from mice vaccinated against AMHR2-CD (Figure 5(c)). Moreover, enhanced gene expression for the natural killer cell receptor, NKRP1A, in tumors from mice vaccinated against AMHR2-CD (Figure 5(c)), implies that NK cells may also play a role in the observed tumor immunity perhaps as a result of recruitment through IFNγ-dependent CXCR3 signaling [41] or through an IL-17/CCL2 recruitment mechanism [42]. Thus, CD4+ T cells may also mediate upregulated expression of angiostatic chemokines such as CXCL9, CXCL10, and CXCL14 that are capable of inhibiting tumor growth (reviewed in [40]) or may downregulate expression of the chemokine receptor CXCR2 or its many ligands that promote angiogenesis (reviewed in [43, 44]). Studies are currently underway to distinguish the underlying mechanism(s) involved in the therapeutic efficacy of AMHR2-CD vaccination against EOC.

5. Conclusion

In several mouse models of EOC, a single vaccination against AMHR2-CD is sufficient to provide effective immune control over the growth of ovarian tumors. Notably, this vaccine-induced tumor immunity occurs in the absence of any severe autoimmune consequences. Thus, AMHR2 vaccination may be useful in controlling the more malignant forms of human ovarian cancer.

Abbreviations

AMH:	Anti-Müllerian hormone
AMHR2:	Anti-Müllerian hormone receptor 2
AMHR2-CD:	Anti-Müllerian hormone receptor, type II-cytoplasmic domain
AIRE:	Autoimmune regulator transcription factor
CA-125:	Cancer antigen 25
CTAG1B or NY-ESO-1:	Cancer-testis antigen 1
CFA:	Complete Freund's adjuvant

cpm: Counts per minute
DC: Dendritic cell
EOC: Epithelial ovarian cancer
HPLC: High performance liquid chromatography
HRP: Horse radish peroxidase
HER2/neu: Human epidermal growth factor receptor tyrosine kinase
IFNγ: Interferon gamma
IPTG: Isopropyl β-D-1-thiogalactopyranoside
NKR-P1A: Natural killer cell lectin-like receptor subfamily B, member 1
LNC: Lymph node cells
MHC: Major histocompatibility complex
mTECs: Medullary thymic epithelial cells
MOSEC: Mouse ovarian surface epithelial cells
Ni-NTA: Nickel-nitrilotriacetic acid
NK: Natural killer
OS: Overall survival
OVA: Ovalbumin
qRT-PCR: Quantitative real-time RT-PCR
SV40-Tag: Simian virus 40 large T antigen
TCR: T cell receptor
TGFβ: Transforming growth factor β
TILs: Tumor infiltrating lymphocytes
TAA: Tumor associated antigen
TNFα: Tumor necrosis factor alpha.

Conflict of Interests

This work was supported by a grant from Shield Biotech, Inc., Cleveland, OH, as privately owned company. Vincent K. Tuohy is the primary inventor of vaccines that have been licensed to Shield Biotech, Inc., and is the Chief Science Officer of Shield Biotech, Inc. As such, Vincent K. Tuohy may in the future receive commercialization revenues for these technologies. All other authors declare that there is no conflict of interests.

Authors' Contribution

Cagri Sakalar, Suparna Mazumder, Cengiz Z. Altuntas, Justin M. Johnson, Robert Aguilar, Sathyamangla V. Naga Prasad, and Vincent K. Tuohy were involved in the development of methodology and in the acquisition of data. Cagri Sakalar, Suparna Mazumder, Ritika Jaini, Denise C. Connolly, and Vincent K. Tuohy were involved in the analysis and interpretation of the data. Cagri Sakalar, Suparna Mazumder, Denise C. Connolly, and Vincent K. Tuohy participated in the writing, review, and revision of the paper. Vincent K. Tuohy was instrumental in the conception, experimental design, and overall supervision of the project.

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Review Article

Immune Checkpoint Modulation in Colorectal Cancer: What's New and What to Expect

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Colorectal cancer (CRC), as one of the most prevalent types of cancer worldwide, is still a leading cause of cancer related mortality. There is an urgent need for more efficient therapies in metastatic disease. Immunotherapy, a rapidly expanding field of oncology, is designed to boost the body's natural defenses to fight cancer. Of the many approaches currently under study to improve antitumor immune responses, immune checkpoint inhibition has thus far been proven to be the most effective. This review will outline the treatments that take advantage of our growing understanding of the role of the immune system in cancer, with a particular emphasis on immune checkpoint molecules, involved in CRC pathogenesis.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide, with almost 1.4 million new cases in 2012 [1]. Patient survival is highly dependent on the tumor stage at the time of diagnosis. Only 40% of CRC cases are diagnosed at an early stage and approximately 50% of recently diagnosed patients will progress to metastatic cancer [2]. The overall 5-year survival of CRC patients is close to 65% ranging from 90% for patients with localized disease to 70% and 13% for patients with localized lymph node metastases or organ metastases, respectively [2]. Although surgery remains the cornerstone in the treatment of this disease, 30–40% of patients have locoregionally advanced or metastatic disease that cannot be cured by surgery alone [3]. Hence, patients at increased risk of disease recurrence and patients with metastatic disease receive adjuvant chemotherapy. Despite the recent progress in diagnosis and treatment, including the introduction of targeted therapies, the prognosis of these advanced CRC remains poor [4].

Advances in molecular biology have helped elucidate some of the genetic mechanisms leading to colorectal carcinogenesis. Most CRC cases are due to sporadic genetic and/or epigenetic changes, but up to 10–20% of all CRC cases have a familial component [2]. There are three major molecular mechanisms that cause aberrant gene expression in CRC: microsatellite instability (MSI), chromosomal instability (CIN), and the CpG island methylator phenotype (CIMP) [2, 5]. Accumulating evidence suggests that tumor progression is governed not only by genetic changes intrinsic to cancer cells but also by environmental factors. Hence, in addition to genetic mutations and TNM staging, a quantitative assessment of immune cells that infiltrate the tumor tissue and peritumoral areas has been proposed as an independent outcome predictor [4]. Increased understanding of the immune tumor microenvironment has allowed for investigation into novel immune-based biomarkers and the development of new agents that target immune pathways for therapy [6]. Among the most promising approaches is the blockade of immune checkpoint molecules to activate antitumor

immunity [7]. Therefore, this review will outline the treatments that take advantage of our growing understanding of the role of the immune system in cancer, particularly highlighting immune checkpoint blockade in CRC.

2. Antitumor Immunity in CRC

2.1. Immune Surveillance and Immunoediting. Through immune surveillance, the body can effectively recognize and eliminate cancerous cells prior to clinical expression [6, 8]. In humans, the role of immune surveillance was first suspected with the observation of increased occurrence of cancer in patients with immunodeficiency. This concept of immune surveillance has long been questioned until it was finally demonstrated in animal models by Shankaran et al. [9]. The selection pressure exerted by the immune system on tumor cells allows resistant clones to escape immune surveillance in a process known as immunoediting [6, 8]. This reciprocal relationship between immune cells and cancer cells occurs in three phases: the immune surveillance period, the latency period, corresponding to a state of equilibrium, and the phase of immune escape, allowing tumor progression and clinical expression [8]. Hence, this complex interaction between tumor cells and the local immune response results in a balance between tumor-promoting and tumor-controlling effects and calls for a close collaboration between cells of the innate immune system and cells of the adaptive immune system [3].

2.2. Innate Immunity. Innate immunity is the first line of defense for the antitumor immune system. Innate immune cells have specialized surface receptors that recognize tumor-specific antigens on cancer cells. Recognition initiates an inflammatory cascade leading to antigen presentation by dendritic cells (DCs) and macrophages to T cells, activating an adaptive immune response. Basically, the innate immune system recognizes tumor-specific antigens on the surface of cancer cells in a similar way as the recognition of non-self-pathogens [6].

Natural killer (NK) cells are one of the key cell types involved in immune surveillance [6]. They do not express antigen specific receptors but are able to eliminate neoplastic cells in the absence of certain major histocompatibility complex (MHC) molecules on target cells [3, 10]. In addition, NK cells may exert a cytotoxic effect against cancer cells through other mechanisms such as antibody dependent cell mediated cytotoxicity (ADCC) and secretion of cytokines, including interferon- (IFN-) γ, leading to the activation of other inflammatory cells, including macrophages and DCs (see below) [6, 10]. In CRC, an extensive intratumoral infiltration of NK cells has been reported to be associated with a better prognosis [11, 12].

Natural killer T (NKT) cells share characteristics of both T cells and NK cells by coexpressing $\alpha\beta$ T cell receptor and a variety of molecular markers that are typically associated with NK cells. NKT cells recognize glycolipid antigens like α-galactosylceramide presented by CD1d, an MHC class I-like molecule that binds self and foreign (glycol) lipids. When activated, NKT cells secrete abundant proinflammatory cytokines (such as interleukin- (IL-) 2, IFN-γ, tumor necrosis factor- (TNF-) α, and IL-4) and effector molecules involved in cell death (such as perforin, Fas ligand, and TRAIL). Similar to NK cells, increased tumor infiltration of NKT cells seems to be associated with a better prognosis in CRC [8].

Recruited macrophages and DCs phagocytose tumor cells and can then present tumor-associated antigens (TAAs) on their surface [6]. DCs form a network of antigen-presenting cells (APCs) that shape immune responses by linking innate and adaptive immunity. In order to instruct naïve T cells into the required functional profile, DCs must present tumor antigens via MHC class I and II molecules, express costimulatory ligands, and secrete inflammatory mediators such as IL-12 or type I IFNs [13]. Macrophages at their turn have Fc receptors on their surface and mediate ADCC. Tumor-infiltrating macrophages (TIM) can be divided into two subtypes. M1 TIM secretes high levels of nitric oxide synthase and proinflammatory molecules (IL-6, IL-12, IL-13, and TNF-α) and promotes adaptive immunity through increased expression of MHC and costimulatory molecules [8, 10]. In contrast, M2 TIM produces arginase and immunosuppressive cytokines [IL-10 and transforming growth factor- (TGF-) β and prostaglandin E2] and promotes angiogenesis via production of vascular endothelial growth factor (VEGF) thereby promoting tumor progression. Tumor-associated macrophage (M2-polarized) infiltration into the tumor microenvironment is generally considered a poor prognostic marker in several tumor types. Conversely, their role in CRC is controversial with numerous studies indicating a better outcome in CRC patients [14–16], while others state the opposite [17].

2.3. Adaptive Immunity. Adaptive immunity is responsible for a long-term specific antitumor immune response, including immune memory related to a prior immune challenge [8, 10]. Briefly touching upon adaptive immune cells, B cells play a major role in humoral adaptive immunity and are involved in sustaining long-term immunity [3, 10]. In addition, tumor-infiltrating B cells can sustain and enhance T cell responses by producing antibodies, stimulatory cytokines, and chemokines in addition to functioning as a local APC.

T cells recognize the signaling complex comprised of $\alpha\beta$ T cell receptor dimer, CD4 or CD8 accessory molecules, and CD3 along with the peptide antigen presented in the context of MHC class I and II molecules [3, 10]. $CD4^+$ T cells recognize class II MHC molecules presented on APC, whereas $CD8^+$ T cells identify class I MHC molecules expressed on several cell types [10]. Activation of T cells requires 3 signals: recognition of tumor cell antigen, activation of costimulatory molecules (CD80/CD28 and CD40/CD40L), and binding of cytokines (IL-1, IL-2, IL-6, IL-12, and IFN-γ) [8]. Upon activation, $CD4^+$ T cells can modulate the antitumor immune response. Depending on the cytokine profile produced by the effector cells, $CD4^+$ T cells are subdivided in different T helper (Th) subsets, each secreting specific cytokines [3]. Th1 cells secrete cytokines such as IL-2 and IFN-γ which promote antitumor immune response by cytotoxic T lymphocytes.

In contrast, Th2 cells secrete IL-4, IL-5, and IL-13, promote IgE synthesis, and are believed to favor tumor growth [8, 10]. The most recent addition to effector Th subsets is Th17 cells which develop from naïve $CD4^+$ T cells in the presence of TGF-β, IL-6, and IL-1β and are maintained for a long term in the presence of IL-21 and IL-23. In addition to producing IL-17A, Th17 cells can produce IL-17F, IL-21, IL-22, IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [18]. Th17 cells play a complex and controversial role in tumor immunity, either promoting or suppressing tumor growth depending on the malignancy and on the therapeutic intervention investigated. Recent findings also point to significant alterations in Th17 cells due to their interplay with cytotoxic $CD8^+$ T cells and regulatory T lymphocytes (Tregs) within the tumor microenvironment [19].

Tregs, of which the best characterized subpopulation expresses CD4, CD25, and Foxp3, function as checkpoint regulators to maintain immune self-tolerance and suppress immune effects against self-antigens [8, 10]. This means that TAAs themselves may induce an increased number of intratumoral Tregs in varying tumor types, including CRC, supporting a role for Tregs in cancer-induced immunosuppression. Hence, targeting Tregs might have an important impact on immunotherapeutic anticancer strategies and the clinical outcome of cancer patients [3]. Activated $CD8^+$ T cells can expand clonally and differentiate into "killer lymphocytes" which will recognize and lyse tumor cells using granule exocytosis and formation of FAS ligand. While most cytotoxic T lymphocytes (CTLs) die through apoptosis following effectuation of their killer function, some become long-lived memory cells [3]. Pronounced lymphocyte infiltration has been described in CRC, is more present in MSI tumors, and is reported to be associated with a better clinical course [20]. T cell activation is regulated by a balance between costimulatory and inhibitory signals (i.e., immune checkpoints). Under normal physiological conditions, immune checkpoints are crucial for the maintenance of self-tolerance. Conversely, tumors are capable of deregulating the expression of these immune checkpoint proteins as an important immune resistance mechanism [21].

3. Immune Checkpoints in CRC

A major turning point in cancer immunotherapy came with the clinical application of antibodies that block immune checkpoints [22]. Blockade of these inhibitory coreceptors and pathways, which restrain T cell functions in normal physiologic settings and are being exploited by tumors, might "release the brakes" on immune responsiveness leading to tumor elimination [23]. On the other hand, numerous immune checkpoints that enable "stepping on the gas" of immune responsiveness have been identified. In this section we will discuss emerging immune checkpoints in CRC pathogenesis (Figure 1).

3.1. PD-1/PD-L1. Programmed death-1 (PD-1, also known as CD279) is a coinhibitory receptor that is inducibly expressed on $CD4^+$ T cells, $CD8^+$ T cells, NKT cells, B cells, and monocytes/macrophages [24]. Known ligands of PD-1 include PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-L1 is constitutively expressed on a wide variety of immune cells and nonimmune cells, whereas for PD-L2 this expression can be induced, depending on microenvironmental stimuli [25]. This pathway has been implicated in tumor immune evasion due to the upregulation of PD-1 on tumor-infiltrating lymphocytes (TILs) and increased expression of its ligands on tumor cells, leading towards suppression of tumor-specific $CD8^+$ T cells. Furthermore, this pathway has been associated with T cell exhaustion in cancer as defined by impairment of proliferation, cytokine production, and cytotoxicity [26]. To overcome this immune suppression, blocking monoclonal antibodies (mAbs) against PD-1 and PD-L1 are emerging and have shown durable responses in metastatic solid tumors.

A role for this pathway in CRC pathogenesis was first shown by the correlation of single nucleotide polymorphisms in the PD-1 gene with CRC in a Chinese population [27] as well as with colon cancer in Iranians [28]. Thereafter, PD-1 was shown to be markedly upregulated on $CD8^+$ T cells in the tumor microenvironment of CRC specimens in comparison to $CD8^+$ tumor-free lymph nodes. Moreover, these $PD1^+CD8^+$ T cells in the tumor microenvironment were associated with the impairment of cytokine and perforin production [26]. Interestingly, the expression level of PD-L1 on CRC seemed to be the crucial player in this impairment of cytokine production [26]. In addition, using immunohistochemistry (IHC), Hua et al. revealed an inverse relationship between the expression of PD-L1 on CRC cells and T cell density in the tumor microenvironment [29]. Next to the reduction in T cells, an expansion of Tregs could be found, marked by the high number of $Foxp3^+$ cells and a strong correlation between $PDL\text{-}1^+$ tumor cells and worse prognosis [29]. Also, in peripheral blood from postsurgical CRC patients, PD-1 expression could be demonstrated on both $CD4^+$ and $CD8^+$ T cells and again marked impaired T cell function [30]. Based on these data, the blockade of PD-1/PD-L1 interaction has been proposed as a therapeutic strategy in CRC. Unfortunately, in a clinical setting, no objective clinical responses of anti PD-1 therapy (BMS936558/Nivolumab/MDX-1106) were observed in 19 CRC patients [31]. Also, in 2012, no response to therapy was seen in 18 CRC patients, using an antagonistic PD-L1 antibody (BMS936559/MDX-1105) [32]. Furthermore, a study by Droeser et al. demonstrated an association of PD-L1 expression with improved survival in CRC specimens [33]. Moreover, a significant correlation between PD-L1 overexpression, infiltration of $PD\text{-}1^-CD8^+$ lymphocytes, and IFN-γ gene expression was observed. Remarkably, this correlation could only be demonstrated in a subset of CRC patients, marked by mismatch repair (MMR) proficient tumors, whereas no association was found in MMR-deficient CRC, also known as MSI [34]. The idea that immune checkpoint blockade could be more effective in MSI CRC was further investigated by a small phase 2 trial of Pembrolizumab, another fully human mAb targeting PD-1. Indeed, this study showed that MMR status predicted clinical benefit of immune checkpoint blockade with Pembrolizumab, with enhanced responsiveness in MSI CRC [35]. In addition, Nivolumab (MDX-1106) was tested in patients

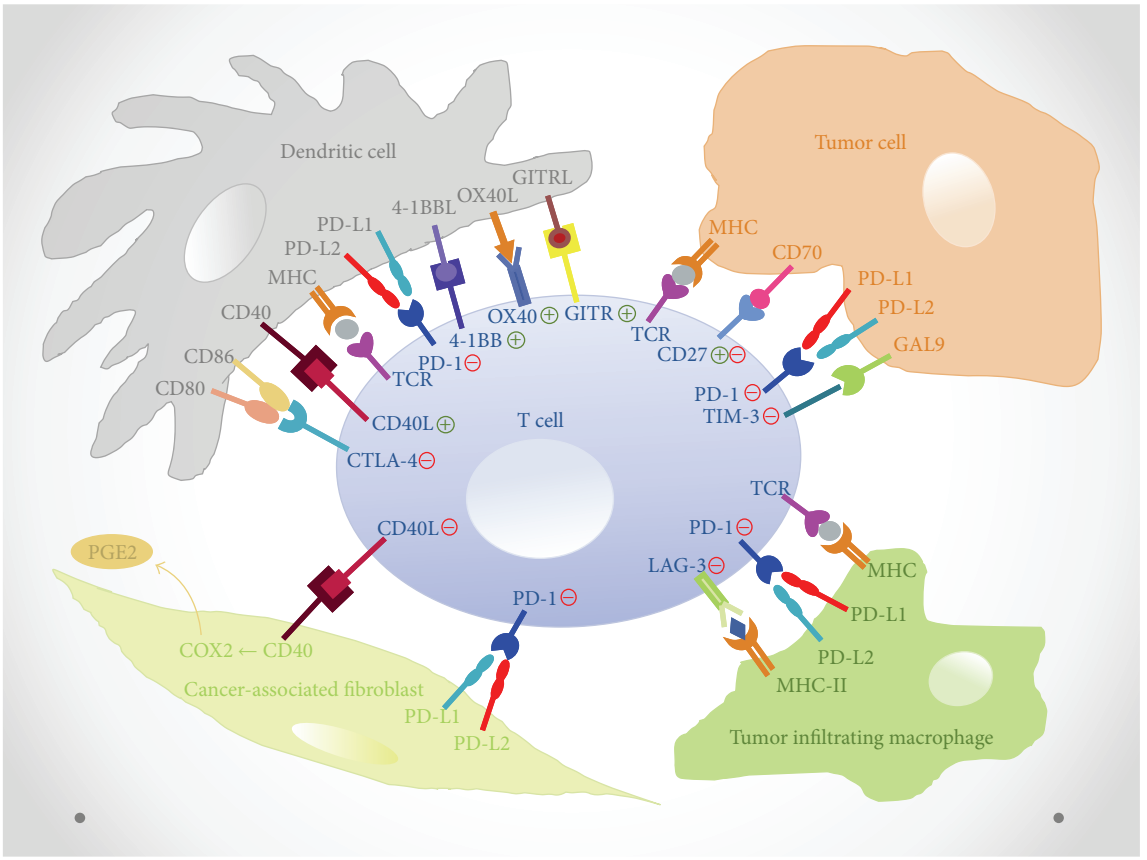

Figure 1: Overview of immune checkpoint molecules involved in CRC pathogenesis. CD, cluster of differentiation; COX2, cyclooxygenase-2; CTLA-4, cytotoxic T lymphocyte antigen-4; GAL9, Galectin-9; GITR, glucocorticoid-induced TNFR-related protein; LAG-3, lymphocyte activation gene-3; MHC, major histocompatibility complex; PD-1, programmed death-1; PD-L, programmed death ligand; PGE2, prostaglandin E2; TCR, T cell receptor; TIM-3, T cell immunoglobulin and mucin containing protein-3.

with advanced treatment-refractory solid tumors, including 14 CRC patients of which one patient achieved a complete response (CR) with no evidence of disease recurrence after three years [23]. Likewise, further studies on the tumor of this patient demonstrated microsatellite instability [36]. The importance of the MMR status for response to therapy is getting more and more clear, demonstrated by the multiple active clinical trials with anti-PD-1 (AMP-224, PDR001, Nivolumab, Pembrolizumab, REGN2810, BGB-A317, and MEDI0680) and anti-PD-L1 therapy (MEDI4736, MDX-1105, Avelumab, and MPD-L3280A) enrolling more patients with MSI status (Table 1). Next to the growing landscape of mAbs, targeting the PD-1/PD-L1/L2 axis in monotherapy, different combination strategies are emerging in these trials using other immune checkpoint inhibitors (Ipilimumab and MEDI4736), immunostimulatory molecules (Denenicokin, RO6895882, Lirilumab, and PF-05082566), targeted therapies (Cobimetinib and Avastin), or conventional therapies (stereotactic body radiation, hypofractionated radiotherapy, and cyclophosphamide) (Table 1).

3.2. CTLA-4/B7. Another molecule involved in T lymphocyte inhibition is cytotoxic T lymphocyte antigen-4 (CTLA-4), expressed on the surface of T lymphocytes. CTLA-4 has similar binding affinities for B7-1 (CD80) and B7-2 (CD86) costimulatory receptors on APC and this interaction transmits inhibitory signals to attenuate T cell activation by competing for B7 ligands with its homologue, CD28 [24]. Therefore, CTLA-4 is an interesting target to block with monoclonal antibodies. One such example is Ipilimumab, currently FDA approved for first-line and second-line treatment of metastatic malignant melanoma. Here, Ipilimumab has been shown to reinvigorate the antitumor immune response by binding CTLA-4 and thereby preventing it from binding to its ligands and reducing the inhibition of CD28/B7 T cell activation. Next to the inhibition of T cell activation, this also resulted in the reduction of Tregs. Since Treg accumulation has been linked with poor outcome in CRC, this might be an interesting therapeutic strategy for CRC [37].

Similar to PD-1, a role of CTLA-4 in CRC development was suggested by multiple groups, showing associations of

Table 1: Clinical trials testing immune checkpoint modulators in colorectal cancer (according to https://www.clinicaltrial.gov/, 19th of June 2015).

Target	Compound	NCT tracker	Phase	Tumor type	Status	Combination (target)
Antagonistic monoclonal antibodies						
PD-1	**AMP-224** <*Amplimmune*	NCT01352884	I	Advanced solid tumors or CTCL	Completed	
		NCT02298946	I	mCRC	Recruiting	+ Stereotactic body RT + Cyclophosphamide
	PDR001 <*Novartis*	NCT02404441	I II	Advanced malignancies incl. only PD-L1$^+$ MSI-H CRC	Recruiting	
		NCT01629758	I	Locally advanced or metastatic solid tumors	Completed	+ Denenicokin (IL-21)
	Nivolumab BMS-936558 MDX-2206 ONO-4538 <*Bristol-Myers Squibb*	NCT02060188	II	Recurrent and mCRC: MSI-H and MSI-L	Recruiting	+ Ipilimumab (CTLA-4)
		NCT02408861	I	HIV-associated solid tumors	Not yet recruiting	+ Ipilimumab (CTLA-4)
		NCT00836888	I	Advanced malignant solid tumors in Japan	Not yet recruiting	
		NCT01714739	I	Advanced solid tumors	Recruiting	+ Lirilumab (KIR)
		NCT02054806	I	Biomarker-positive solid tumors	Recruiting	
		NCT02179918	I	Advanced solid tumors	Recruiting	+ PF-05082566 (CD137)
	Pembrolizumab MK-3475 <*Merck*	NCT02332668	I/II	Advanced Melanoma; advanced relapsed PD-L1$^+$ malignancies	Recruiting	
		NCT01876511	II	MSI-H (non)-CRC	Recruiting	
		NCT02460198	II	Previously treated locally advanced unresectable/MSI-H mCRC	Not yet recruiting	
	REGN2810 <*Regeneron*	NCT02383212	I	Advanced malignancies	Recruiting	+ Hypofractionated RT + Cyclophosphamide
	BGB-A317 <*BeiGene*	NCT02407990	I	Advanced cancers	Recruiting	
	MEDI0680 AMP-514 <*AstraZeneca*	NCT02013804	I	Advanced cancers	Recruiting	
		NCT02118337	I	Advanced cancers	Recruiting	+ MEDI4736 (PD-L1)
PD-L1	**MEDI4736** <*AstraZeneca*	NCT01693562	I/II	Solid tumors	Recruiting	
		NCT02227667	II	Advanced CRC	Recruiting	
	MDX-1105 BMS-936559 <*Bristol-Myers Squibb*	NCT00729664	I	Relapsed/refractory solid tumors (incl. CRC)	Active, not recruiting	
	Avelumab MSB0010718C <*MerckKGaA and Pfizer*	NCT01943461	I	Metastatic/locally advanced solid tumors	Recruiting	
		NCT01772004	I	Solid tumors	Recruiting	
		NCT01375842	I	Locally advanced/metastatic solid tumors incl. CRC	Recruiting	
	MPD-L3280A MSB0010718C <*Roche*	NCT01633970	I	Locally advanced or metastatic solid tumors (incl. >10 patients with CRC)	Recruiting	+ Avastin (VEGF) + Chemotherapy
		NCT02350673	I	Metastatic/locally advanced solid tumors	Not yet recruiting	+ RO6895882 (IL-2)
		NCT01988896	I	Metastatic/locally advanced solid tumors incl. KRAS-mutant mCRC	Not Yet recruiting	+ Cobimetinib (MEK)

Table 1: Continued.

Target	Compound	NCT tracker	Phase	Tumor type	Status	Combination (target)
CTLA-4	**Ipilimumab** MDX-010 YERVOY <*Bristol-Myers Squibb*	NCT01750983	I	Advanced or metastatic cancer	Recruiting	+ Lenalidomide
		NCT02239900	I/II	Advanced solid tumors with spread to liver, lung, or adrenal gland	Recruiting	+ Stereotactic body radiation
	Tremelimumab Ticilimumab CP-675,206 <*Pfizer*	NCT00313794	II	mCRC	Completed	
		NCT01975831	I	Advanced solid tumors (incl. CRC)	Recruiting	+ MEDI4736 (PD-L1)
		NCT02261220	I	Advanced solid tumors	Recruiting	+ MEDI4736 (PD-L1)
LAG-3	**BMS-986016** <*Bristol-Meyers Squibb*	NCT01968109	I	Solid tumors	Recruiting	+ Nivolumab (PD-1)
	LAG-525 <*Novartis*	NCT02460224	I II	Advanced solid tumors (incl. PD-L1$^+$CRC MSI-H)	Not yet recruiting	+ PDR001 (PD-1)
CD70	**ARGX-110** <*arGEN-x*	NCT01813539	I	Refractory or relapsing CD70$^+$ malignancies	Recruiting	
Agonistic monoclonal antibodies						
CD27	**Varlilumab** CDX-1127 <*Celldex*	NCT01460134	I	Solid tumors (incl. CRC)	Recruiting	
		NCT02335918	I/II	Advanced refractory solid tumors (incl. CRC)	Recruiting	+ Nivolumab (PD-1)
CD134 (OX40)	**MEDI6469** <*AgonOx*	NCT02318394	I	Recurrent or metastatic solid tumors	Recruiting	
		NCT02205333	I/II	Advanced solid tumors/aggressive B-cell lymphomas	Recruiting	+ Tremelimumab (CTLA-4) + MEDI4736 (PD-L1) + Rituximab
	MEDI6383 <*AgonOx*	NCT02221960	I	Advanced solid tumors	Recruiting	
	MOXR0916 RG7888 <*Genentech Inc.*	NCT02219724	I	Metastatic/locally advanced solid tumors	Recruiting	
		NCT02410512	I	Locally advanced, recurrent, or metastatic incurable solid tumors	Recruiting	+ MPL3280A (PD-L1)
Agonistic monoclonal antibodies						
GITR	**TRX518** <*Tolerx*	NCT01239134	I	Solid tumors/malignant melanoma	Recruiting	
	MK-4166 <*Merck*	NCT02132754	I	Solid tumors	Recruiting	+ Pembrolizumab (PD-1)
CD137 (4-1BB)	**Urelumab** BMS-663513 <*Bristol-Meyers Squibb*	NCT01471210	I	Advanced solid tumors/B-cell NHL	Recruiting	
		NCT02110082	I	CRC/head and neck cancer	Recruiting	+ Cetuximab (EGFR)
		NCT02253992	I/II	Advanced solid tumor/advanced B-cell NHL	Recruiting	+ Nivolumab (PD-1)
	PF-05082566 <*Pfizer*	NCT02444793	I/II	Advanced/metastatic solid tumors	Recruiting	+ Mogamulizumab (CCR4)
CD40	**CP-870,893** <*Pfizer*	NCT02225002	I	Advanced solid tumors	Completed	
		NCT00607048	I	Metastatic solid tumors	Completed	+ Paclitaxel/Carboplatin
	RO7009789 <*Roche*	NCT02304393	I	Metastatic/locally advanced solid tumors	Recruiting	+ MPD-L3280A (PD-L1)
	ADC-1013 <*AlligatorBioscience*	NCT02379741	I	Advanced solid tumors	Recruiting	
	ChiLob 7/4 <*Southampton, UK*	NCT01561911	I	CD40$^+$ solid tumors/refractory DLBCL	Completed	

Table 1: Continued.

Target	Compound	NCT tracker	Phase	Tumor type	Status	Combination (target)
	SEA-CD40 <*Seattle genetics*	NCT02376699	I	Advanced metastatic tumors/unresectable solid malignancies	Recruiting	

PD-1, programmed death-1; PD-L1, programmed death ligand-1; CTLA-4, cytotoxic T lymphocyte antigen-4; CD, cluster of differentiation; LAG-3, lymphocyte activation gene-3; GITR, glucocorticoid-induced tumor necrosis factor receptor-related protein; CTCL, cutaneous T cell lymphoma; CRC, colorectal cancer; mCRC, metastatic CRC; MSI, microsatellite instability; MSI-H, MSI-high; MSI-L, MSI-low; HIV, human immunodeficiency virus; KRAS, Kirsten rat sarcoma viral oncogene; NHL, non-Hodgkin lymphoma; DLBL, diffuse large B cell lymphoma; IL, interleukin; KIR, killer-cell immunoglobulin-like receptor; RT, radiotherapy; VEGF, vascular endothelial growth factor; MEK, mitogen-activated protein kinase kinase; EGFR, epidermal growth factor receptor; CCR4, C-C chemokine receptor type 4.

CTLA-4 single nucleotide polymorphisms and the risk of developing CRC [27, 38–40]. CTLA-4 49A/G polymorphism came forth as a major player in CRC development. It was also demonstrated that CTLA-4 is expressed at considerably higher levels in MSI tumors as compared to MSS [41]. Here, the expression of CTLA-4 was found not only on TILs intercalated within the epithelial component of the tumor but also in the surrounding tumor stroma and at the invasive front of the tumor. Of particular interest is also the expression of CTLA-4 on multiple subsets of Tregs. First, a significant increase of activated Tregs ($CD45RA^{-}Foxp3^{+}$ T cells) in peripheral blood and cancer tissue of patients with colon cancer was demonstrated with significantly higher levels of CTLA-4 [42]. Second, accumulation of $CCR4^{+}CTLA\text{-}4^{+}$ regulatory T cells was found in colon adenocarcinomas as well as an increase in $CTLA\text{-}4^{+}$ conventional T cells, susceptible to immune regulation in the tumor-associated mucosa [43]. Finally, the presence of a potent suppressive $CD4^{+}Foxp3^{-}$ T cell population was revealed within the colorectal tumor regulatory landscape by comparison of healthy colon, colorectal tumor samples, and matched blood from CRC patients [44]. These $CD4^{+}Foxp3^{-}$ T cells seemed to coexpress immune checkpoints such as LAG-3, PD-1, and CTLA-4 and were able to produce immunosuppressive cytokines such as IL-10 and TGF-β. More importantly, this unique population was 50-fold more suppressive than $Foxp3^{+}$ Tregs. The expression of CTLA-4 on different subsets of regulatory T cells makes this immune checkpoint an interesting therapeutic strategy, which might lead to strengthening of the antitumor immune response in CRC [44]. In this regard, Tremelimumab, a similar antibody to Ipilimumab, has been investigated in a phase II study for patients with refractory metastatic adenocarcinoma of the colon or rectum who failed standard chemotherapy. Surprisingly, only a single patient received a second dose, whereas the remaining 46 patients had disease progression or disease-related death before reaching the planned second dose at 3 months [45]. Because these data do not support further investigation of Tremelimumab as a single agent for the treatment of advanced, treatment-refractory colorectal cancer, phase I trials are now ongoing in combination with MEDI4736, a PD-L1 antagonistic mAb, in patients with solid tumors. Furthermore, phases I and I/II of Ipilimumab are actively recruiting patients with metastatic solid tumors in combination with stereotactic body radiation or Lenalidomide (Table 1).

3.3. TIM-3. T cell immunoglobulin and mucin containing protein-3 (TIM-3) was discovered as a molecule expressed on IFN-γ producing $CD4^{+}$ Th1 and $CD8^{+}$ cytotoxic T cells. Through its ligand, Galectin-9, TIM-3 is believed to play a critical role in inhibiting Th1 responses and inducing cell death [46]. Furthermore, animal models have revealed its role in T cell exhaustion due to the expression of TIM-3, together with PD-1, in the most suppressed or dysfunctional populations of $CD8^{+}$ T cells in hematological as well as solid malignancies. In preclinical models, blocking TIM-3 was able to reinvigorate antitumor activity, comparable to the effect of PD-1 blockade with a greater efficacy through combination of both.

In peripheral blood samples from CRC patients, Xu et al. demonstrated significantly higher levels of circulating $TIM\text{-}3^{+}PD\text{-}1^{+}CD8^{+}$ T cells compared to healthy blood [47]. Also peripheral blood, drawn after surgery, exposed the expression of TIM-3 and PD-1 on $CD8^{+}$ T cells and $CD4^{+}$ T cells. Moreover, the expression of both TIM-3 and PD-1 appeared to relate with the impaired function of these T cells [30]. Likewise, an increase of $Tim\text{-}3^{+}PD\text{-}1^{+}CD8^{+}$ T cells was observed in CRC tissue, when compared to tissues adjacent to the tumor. Interestingly, distinguishing the subset of T cells by the expression of PD-1 demonstrated a significant lower level of IFN-γ production in the $PD\text{-}1^{-}$ subset. Together with the lack of objective responses by PD-1 blockade in a large population of CRC patients (as discussed above), these results suggest TIM-3 as a more dominant inhibitory receptor, restricting T cell responses in CRC patients. In addition, blocking this pathway might restore the impaired cell-mediated immunity after surgical resection. Taken together, these data support the development of TIM-3 inhibitors and hold great promise as single or combined modalities in CRC patients [48].

3.4. LAG-3. Another interesting target for immune checkpoint blockade is lymphocyte activation gene-3 (LAG-3, also known as CD223), a cell surface molecule of the immunoglobulin superfamily. Through its interaction with MHC class II, LAG-3 has been demonstrated to play a pivotal role in negative regulation of T cell proliferation, enabled by its expression on activated T cells, NK cells, B cells, and plasmacytoid DCs [48, 49]. In addition, LAG-3 appears to mitigate Treg function. Indeed, the expression of LAG-3 on $CD4^{+}CD25^{+}$ cells was able to define a subset of cells endowed

with potent suppressor activity [50]. Together with CD49b, the expression of LAG-2 marks highly suppressive human type 1 regulatory T cells (Tr1), a subgroup of Tregs producing IL-10 [51]. It was also recently revealed that exhausted $CD8^+$ T cells can express LAG-3 and that the expression of multiple inhibitory receptors, such as the combination with PD-1, was associated with greater T cell exhaustion. Moreover, simultaneous inhibition of PD-1 and LAG-3 could enhance T effector activity as compared to either molecule alone [52]. Henceforward, clinical trials with LAG-3 inhibitors (LAG-525 and BMS-986016) are now progressing into phase I studies, with or without the combination of PD-1 inhibitors (Nivolumab and PDR001) in patients with advanced solid malignancies (Table 1).

J. Chen and Z. Chen examined 108 CRC tissues and their healthy colorectal mucosa and demonstrated a significant increase in the percentage of $LAG\text{-}3^+/CD49b^+$ cells as compared with peritumoral tissues [53]. The increase of Tr1 cells in tumor tissues suggests a crucial role for this subset of cells in CRC progression and seems to be predictive for poor prognosis. It is therefore not unexpected that clinical trials with LAG-3 inhibitors have been designed to enroll CRC patients (Table 1).

3.5. CD70/CD27. Although expression of CD70, a member of the tumor necrosis factor family, is normally restricted to activated T and B cells and mature dendritic cells, constitutive expression of CD70 in tumor cells has been described [54]. Through its ligand, CD27, the upregulation of CD70 by tumor cells can facilitate evasion of the immune system by three important mechanisms: induction of T cell apoptosis, skewing T cells towards T cell exhaustion, and increasing the amount of suppressive Tregs [55]. Moreover, *in vivo* experiments demonstrated evasion of immune surveillance by recruitment of $CD27^+$ Treg to the tumor site [56]. The role of CD70-mediated immune escape was also demonstrated in non-small cell lung cancer (NSCLC), where $CD27^+$ lymphocytes were found in the tumor microenvironment with a trend towards increased Foxp3 expression and higher CD4/CD8 ratios surrounding $CD70^+$ tumor cells [57]. Although expression of CD70 in tumor cells of colorectal origin has not been published to date, preliminary data of our group showed expression of CD70 in 6/28 CRC biopsies (Jacobs et al., unpublished data). Furthermore, immunohistochemistry on colon biopsies revealed expression of CD70 in 9% of cases (17/194) [58].

These observations, paired with the limited expression profile of CD70 in normal conditions, present an interesting opportunity to target this molecule in CRC. To date, three anti-CD70 immunoglobulins have entered clinical trial of which ARGX-110 is the only one, enrolling solid and hematological $CD70^+$ advanced malignancies in the study (Table 1).

Contrary to the CD70-blocking strategy, other groups focus on the immunostimulatory potential of a CD27-agonistic monoclonal antibody, such as Varlilumab (Table 1). CD27 belongs to the tumor necrosis factor receptor superfamily (TNFRSF) and plays a key role in immunological processes, such as T cell survival, T cell activation, and the cytotoxic activity of NK cells [59]. Furthermore, ligation of CD27 by CD70 has shown stimulatory effects on T cell proliferation, expansion, and survival dependent upon IL-2 autocrine signaling [60, 61]. As stated above, CD27 triggering may also lead to tumor progression through the recruitment of $CD27^+$ Tregs, complicating the use of CD27 as a target for immunotherapy. However, a fully human monoclonal CD27 agonist antibody, CDX-1127, is being evaluated in solid malignancies, with or without the administration of Nivolumab (see Table 1) and seems able to tear apart the inhibitory and costimulatory mechanisms [60]. Moreover, tumor shrinkage in one CRC patient has already been demonstrated in the dose escalation study [62].

3.6. OX40 (CD134). OX40, also known as CD134, is another costimulatory immune checkpoint molecule of the TNFRSF, capable of stimulating therapeutic immune responses. This molecule has been shown to be transiently upregulated on $CD4^+$ and $CD8^+$ T cells after T cell receptor engagement and during antigen specific priming [63]. Its ligand, OX40L, is presented on activated antigen-presenting cells as well as activated endothelial cells, epithelial cells, and B cells [64]. Furthermore, nonclinical models show OX40 cell surface expression is induced following activation of NK cells with enhanced NK cell activity upon ligation of OX40. Preclinical studies with anti-OX40 agonistic mAb show augmented T cell differentiation, survival, expansion, and cytolytic function [65]. In addition to promoting effector T cell expansion, OX40 agonists have the ability to directly regulate Treg cells by diminishing its inhibitory effects and thereby promoting antitumor $CD8^+$ T cell responses necessary to maintain long-term antitumor immune responses [63].

$OX40^+CD4^+$ TILs have been detected in breast cancer, sarcoma, and melanoma as well as CRC. Indeed, Petty et al. demonstrated high levels of $OX40^+$ lymphocytes in half of primary CRC specimens with a significant correlation towards better survival in the latter [65]. Moreover, OX40 expression levels were the highest inside the tumor and significantly decreased towards the direction of the tumor border and healthy tissue in 39 CRC patients [64]. These results suggest a weakened immune response at the border of the tumor, making it an interesting target for immunotherapy in CRC.

In vivo OX40 agonistic antibodies showed regression of at least 1 metastatic lesion in 12 out of 30 patients after only 1 cycle of treatment [66]. Despite these positive results, it is unlikely that anti-OX40 alone will be sufficient to induce complete response, since antitumor immunity is directed by a dynamic constellation of signals. Therefore, maximizing the therapeutic benefit of OX40 agonists (MEDI6469, MEDI6383, and MOXR0916) will likely depend on the combination with antagonistic Abs, like PD-L1 (MEDI4736 and MPL3280A) and CTLA-4 (Tremelimumab) targeting antibodies (see Table 1) [63].

3.7. GITR. Glucocorticoid-induced TNFR-related protein (GITR, also known as CD357) is a surface receptor molecule that has been shown to be involved in inhibiting the suppressive activity of Tregs and extending the survival of T effector

cells. Therefore, it may hold great promise for the generation of agonistic antibodies. Next to the transient expression on activated $CD4^+$ and $CD8^+$ T cells and the constitutive expression on Tregs, expression has been observed on DC, monocytes, and NK cells [60]. GITRL, its unique ligand, is highly expressed on activated APCs and endothelial cells and ligation with GITR appears to provide costimulation of effector T lymphocytes [67]. Preclinical studies have shown that GITR agonistic agents (like DTA-1) can mediate tumor regression in animal models in part based on a unique mechanism causing Tregs to lose lineage stability, reducing their suppressive influence over the tumor microenvironment [68]. Furthermore, T cell stimulation through GITR attenuates Treg-mediated suppression or enhances tumor-killing by $CD4^+$ and $CD8^+$ effector T cells. Furthermore, a synergistic effect was shown after coadministration of anti-GITR with adoptive T cell transfer and anti-CTLA-4 mAbs, leading to eradication of more advanced tumors [60, 69, 70]. This latter effect was confirmed in murine models bearing fibrosarcoma or CRC.

In CRC patients with liver metastasis, the tumor-specific T cell response is comprised by high numbers of activated Tregs, expressing high levels of GITR and inducible T cell costimulator (ICOS) [67]. Moreover, treatment with soluble GITRL was able to inhibit Treg-mediated suppression, preventing hyporesponsiveness of effector T cells [67]. Although to date preclinical data supporting the use of agonistic GITR mAb for immunotherapeutic interventions in CRC are scarce, two GITR agonistic antibodies (TRX518 and MK-4166) are being investigated in a phase I setting, with or without the addition of a PD-1 inhibitor (Pembrolizumab) (Table 1).

3.8. 4-1BB (CD137). 4-1BB, also known as CD137, is a member of the TNFRSF and is widely known as a T cell costimulatory receptor induced after T cell antigen recognition. 4-1BB binds a high-affinity ligand, 4-1BBL, present on APCs to transduce signals for T cell growth and differentiation. Although both $CD4^+$ and $CD8^+$ T cells express 4-1BB at similar levels, upon activation, signals through 4-1BB are more biased towards $CD8^+$ T cells [71]. Besides its expression on T cells, 4-1BB is expressed, albeit at low levels, on a multitude of cells of the hematopoietic lineage including B cells, regulatory T cells, NKs, NKTs, DCs, mast cells, and early myeloid progenitor cells [72]. Also, a number of studies showed expression of 4-1BB on a wide range of tumor cells [71, 72]. The broad range of 4-1BB expression on multiple cell types makes this receptor a dual-edged sword in the fight against cancer as 4-1BB agonists might elicit strong antitumor responses from a myriad of cell types, however, sometimes at the cost of off-target immune pathology [72].

Cepowicz et al. studied the expression of 4-1BB in peripheral blood samples of 72 patients with primary CRC and demonstrated a direct correlation of 4-1BB positivity and CRC stage as well as invasion depth [73]. Furthermore, an increase in 4-1BB (as well as CD134) was found in peripheral blood taken after surgical resection for CRC, which might be due to increased IL production after elimination of a tumor. On the other hand, expression of its ligand was shown to be lower in cancerous colon tissue compared with paired normal tissue [74]. Suppressed levels of 4-1BBL might indicate the involvement of this pathway in immune escape of colon tumors by the decreased interactions of T cells with tumor cells and macrophages. Interestingly, patients harboring this increased expression of 4-1BB were shown to have high soluble 4-1BB levels in their plasma [74]. Interaction of this soluble 4-1BB with 4-1BBL has been shown to control T cell function by inhibiting the ligation of 4-1BBL with 4-1BB and therefore these results suggest a possible feedback loop to reduce further activation of T cells [75]. Interestingly, this could not be shown in rectal cancerous tissue pointing towards different carcinogenesis of CRC based on the tumor location. Furthermore, the beneficial effects of 4-1BB agonism for the treatment of CRC with hepatic metastases have already been demonstrated in animal models [76, 77].

Currently, two 4-1BB agnostic antibodies (Urelumab and PF-05082566) have entered the clinical setting, enrolling patients with advanced solid tumors or B cell non-Hodgkin lymphoma. For Urelumab, dose escalation data revealed an acceptable toxicity rate across a wide dose range (0.3–15 mg/kg) with increasing percentages of circulating activated $CD8^+$ and $CD4^+$ T cells following a single treatment [78]. Based on these promising results, a phase II study was designed in patients with metastatic melanoma. Surprisingly, this study was terminated due to fatal hepatotoxicity. Henceforward, further trials are mainly focused on low-dose therapies in combination with approved mAbs (Table 1) [79]. For PF-05082566, no significant elevations in liver enzymes and no dose-limiting toxicities have occurred to date [80]. Moreover, PF-05082566 was well tolerated in a first clinical setting with stable disease, observed in 6 out of 27 patients treated. Although these agonistic antibodies hold promise in monotherapy, an interesting combination strategy of 4-1BB agonistic mAb with monoclonal antibodies equipped to induce ADCC was shown [81]. Of interest here is Cetuximab, a human mouse chimeric IgG1 mAb used to treat EGFR expressing *RAS* wild-type metastatic CRC patients. 4-1BB is upregulated on human NK cells when they encounter antibody-bound tumor cells. Moreover, increased levels of 4-1BB on circulating and intratumoral NK cells were directly correlated to an increase in EGFR-specific $CD8^+$ T cells and the combination with Cetuximab marked clear synergism, shown by the complete tumor resolution and prolonged survival [81, 82]. Also *in vivo* this combination regimen has been launched in a clinical setting, combining Urelumab (4-1BB) with Cetuximab in CRC and head and neck cancer patients. Additionally, the combination of PF-05082566 with Mogamulizumab, another ADCC-mediating antibody targeting CCR4, has been initiated (Table 1).

3.9. CD40. CD40, a final member of the TNFRSF, was initially characterized on B cells and is also expressed on DCs, monocytes, platelets, and macrophages as well as by nonhematopoietic cells such as myofibroblasts, fibroblasts,

epithelial, and endothelial cells. The ligand of CD40, known as CD154 or CD40L, is expressed primarily by activated T cells as well as activated B cells and platelets [83]. CD40/CD40L interactions on activated Th cells enhance antigen presentation and expression of costimulatory molecules, licensing DC to mature and achieve all of the necessary characteristics to effectively trigger T cell activation. In murine models, engagement by CD40L promoted cytokine production and enabled effective T cell activation and differentiation [84]. Except for its expression in cells of hematopoietic origin, expression of CD40 has also been demonstrated in several types of carcinoma cells, rendering them susceptible for apoptosis [85]. Interestingly CD40 expression seems absent on normal epithelium, suggesting that expression confers a growth advantage in early stages of malignant development [86]. It has been suggested that neoplastic growth utilizes the CD40/CD40L pathway independent of the immune system to sustain proliferative capacity and survival. Moreover, this receptor/ligand interaction enables tumors to manipulate both T cell and APC compartments most likely contributing to the establishment of the immunosuppressive tumor microenvironment [83].

In CRC, Georgopoulos et al. were the first to demonstrate expression of CD40 in CRC cell lines and colon cancer, with strong (2/17), moderate (4/17), or weak (11/17) positivity in the tumor cells [87]. CD40L was also detected in a number of primary colorectal carcinomas, suggesting an important role of CD40/CD40L axis in CRC tumor immunity (Baxendale et al., unpublished observations) [86]. Contrary to the importance of CD40 expression in early stages of malignant development, progression of malignancy renders cells susceptible to direct antiproliferative effects and CD40-mediated growth inhibition or apoptosis, leading to loss of CD40 expression [88]. Consequently, the use of CD40 as a prognostic tool has been demonstrated, although further research to elucidate its role in CRC is mandatory [87, 89]. Nevertheless, $CD40^+$ TAM and plasma sCD40 in colorectal cancer tissues have already been found to be favorable prognostic markers [90]. In addition, using membrane bound CD40L, but not soluble agonists, a powerful proapoptotic signal and proinflammatory cytokine production could be triggered in CRC cells [87]. These results suggest that CD40 is a promising therapeutic target for the eradication of CRC tumors.

Preclinical investigations with CD40 agonists have been robust and highlight multiple mechanisms of action to overcome tolerance and drive potent T cell immunity in lymphoma and certain solid tumors. Initial clinical trials of agonistic CD40 mAb have shown clinical activity in the absence of disabling toxicity. However, overall response rates remain 20% or less, proposing that CD40 agonists will be most effectively used in combination with other modalities such as chemotherapy, radiation, and vaccines or with negative checkpoint molecule blockers like anti-CTLA-4 or anti-PD-L1 mAbs [91]. In this regard, the safety of CP-870, 893, a fully human CD40 agonistic mAb with carboplatin and paclitaxel, was assessed in a phase I study. Of the 30 evaluable patients, 6 exhibited partial responses, providing a rationale for phase II studies [92]. To date, four other CD40-agonistic antibodies (ADC-1013, RO7009789, SEA-CD40, and ChiLob 7/4) are enrolled in a phase 1 clinical setting with or without the combination of PD-L1 blocking antibodies (MPD-L3280A).

4. Role of Cancer Associated Fibroblasts in Immunomodulation

Increasing evidence has suggested that antitumor efficacy of cancer immunotherapies could be limited by the presence of cancer associated fibroblasts (CAFs). In CRC, CAFs are the main cellular components of the tumor reactive stroma and play a key role in CRC development enabling the induction of immunosuppressive factors, modulation of the microenvironment to a Th2 phenotype, and inhibition of antigen-specific T cell responses and have been considered the main determinants in metastatic progression [93, 94]. A variety of immune cells aid in this process; for example, monocytes differentiate into a distinct M2 polarized macrophage with poor antigen-presenting capacity and further suppress Th1-adaptive immune responses. Additionally, CAFs are also the principal cells producing extracellular matrix within tumor tissue, providing a physical barrier for the immune attack induced by immunotherapies [93, 95]. Furthermore, CAFs seem able to inhibit the proliferation of activated T cells. Herein, the role of immune checkpoints molecules is becoming more and more clear, such as the expression of PD-L2 on human colonic fibroblasts, resulting in T cell suppression in the gut epithelial mucosa. 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 [25]. Strikingly, Nazareth et al. and colleagues found constitutively high PD-L1 and 2 expression in fibroblasts that were cultured from human NSCLC [96]. Moreover, this expression appeared to be functional, since *in vitro* blocking studies demonstrated that the fibroblasts inhibited IFN-γ production in a PD-L1 and 2 dependent matter. The CD40-CD40L axis appears to be another critical pathway for fibroblast and immune system interaction [97]. Indeed, expression of CD40 has been demonstrated on fibroblasts from human lung, orbit, thyroid, and gingiva. Moreover, during inflammation and in fibrotic conditions, activated T cells, eosinophils, and mast cells displaying CD40 ligand are translocated to sites adjacent to fibroblasts enhancing the inflammatory process by inducing synthesis of cytokine mediators and adhesion molecules [97]. Thereby, activation of the transcription factor NF-kB has been shown, resulting in the secretion of high levels of IL-6 and IL-8 as well as the induction of proinflammatory prostaglandin E2 (PGE2) synthesis by fibroblasts (Figure 1). Interestingly, PGE2 severely inhibits both the acquisition of activating receptors and the release of cytotoxic granules by NK cells, resulting in immune evasion [48]. In contrast to the immunostimulatory potential of an agonistic CD40 mAb, stimulating the CD40/CD40L axis on fibroblast might have detrimental effects on the antitumor response. Therefore, future studies should not merely focus on the expression of immune checkpoint molecules by tumor cells but also take the tumor stroma into account, with a particular focus on CAFs.

5. Role of Known CRC Biomarkers in Immunomodulation

Despite recent development and implementation of personalized cancer medicine based on genetic profiling of individual tumors, patient selection for CRC therapy remains challenging. Lately, there has been an increasing interest in biomarkers to predict future patterns of CRC disease. Several promising candidate markers have been investigated for targeted therapies in CRC, including MSI, *KRAS*, and *BRAF* mutations. Furthermore, Galon et al. reported that the adaptive immune response influences the behavior of human tumors [98]. However, the factors that determine a patient's immune phenotype are unclear, and few systematic analyses have investigated the somatic and germline molecular drivers of immune infiltration [99]. Nevertheless, identification of genetic factors that influence the tumor microenvironment is essential to improve the effectiveness of stratified immunotherapy approaches [99].

5.1. MSI. Lal et al. carried out a bioinformatic analysis of CRC data in The Cancer Genome Project involving two-dimensional hierarchical clustering to define an immune signature [99]. A group of 28 tightly coregulated immune-related genes were identified and termed the Coordinate Immune Response Cluster (CIRC). An important feature of the CIRC signature is that it includes essentially all class II MHC loci, as well as CD4, whereas, in contrast, expressions of class I MHC molecules, CD8B, and granzyme B are all excluded. In addition, CIRC also included the major immune checkpoint molecules, including PD-L1, PD-L2, LAG-3, TIM-3, and CTLA-4. One of the key aims of this study was to examine the somatic factors associated with the immune response in CRC. It was shown that MSI-high (H), which is the molecular fingerprint of a deficient DNA mismatch repair system and linked to a high mutational burden, is associated with a high immune infiltration characterized by Th cells and class II related genes, ranges of chemokines, and immune inhibitory checkpoint molecules. Hence, MSI-H tumors may be particularly amenable to $CD4^+$ cell expansion and adoptive transfer approaches, yet the coordinated expression of checkpoint inhibitor genes observed suggests combination checkpoint blockade therapy may be required to improve efficacy. Similarly, POL (polymerase) mutant tumors, which also have a high mutational burden, were also associated with high CIRC expression.

Likewise, Llosa and colleagues examined the immune microenvironment of primary CRC using IHC, laser capture microdissection/qRT-PCR, flow cytometry, and functional analysis of tumor-infiltrating lymphocytes [41]. It was suggested that MSI represents a classical example of adaptive resistance in which an active immune Th1/CTL microenvironment results in a compensatory induction of checkpoints, including PD-1, PD-L1, CTLA-4, IDO, and LAG-3, which protect the tumor from apoptosis [21, 41]. However, the interface between MSI tumors and T cells seems to be characterized by little expression of PD-L1 on tumor cells despite IFN-γ expression by the T cells. Instead, the T cells infiltrate was interlaced with an abundant PD-L1 positive myeloid cell population that presumably inhibits the T cell response. On the basis of these findings, two clinical trials have been initiated to test PD-1 blockade in patients with MSI-H CRC (see Table 1). Combinations with IDO, LAG-3, CTLA-4, and other checkpoints will likely follow [100].

5.2. KRAS. In contrast to the results on MSI, Lal and colleagues showed that *RAS* mutation predicts for a relatively poor immune infiltration and low inhibitory molecule expression. *KRAS* and *NRAS* mutant CRC had significantly lowered levels of $CD4^+$ T cells [99]. Thus, any immunology-based therapy in *RAS* mutant tumors should take into account this immunologically relatively quiescent status of the tumor microenvironment. In this setting, checkpoint blockade may be less efficacious, highlighting the requirement for novel strategies in this patient group [99]. In addition, Kocián et al. examined the correlations between the *KRAS* mutational status, patterns of tumor-infiltrating immune cells, and the presence of tumor recurrence in a cohort of newly diagnosed CRC patients [4]. They observed a significantly higher proliferation rate in tumors with codon 13 mutations as well as a marked variability in the pattern of tumor-infiltrating immune cells regardless of the mutation type. These patients showed a low level of TILs and a high $CD1a^+$/$CD\text{-}LAMP^+$ tumor-infiltrating DC ratio indicating a high risk of cancer-related death. Because the quantification of immune responses within the tumors indicated a strong predictive role in CRC patients, the combined characterization of genetic features and immune cells might provide the foundation to identify high-risk patients [4].

5.3. BRAF. Finally, activating mutations in *BRAF* have been reported in 5%–15% of CRC cases and are frequently found in MSI-H tumors. While *BRAF* mutation is associated with worse survival in MSS tumors, its role in MSI-H tumors is more controversial. It has been postulated that it is not the *BRAF* mutation itself that confers a poor prognosis but rather the fact that the mutation has different effects depending on the type of genetic pathway in which it is produced [3]. Currently, no data are available on the impact of *BRAF* mutation on the tumor immune landscape of CRC. However, recent evidence indicates that melanomas bearing mutant *BRAF* may also have altered immune responses, suggesting additional avenues for treatment of this patient group [101]. Significant advances in the treatment of melanoma have been made with *BRAF*-targeted therapy, not only leading to significant but short-lived clinical responses in a portion of patients but also leading to immunostimulatory bystander events, which then subside with the emergence of resistance [102]. Combination of *BRAF* inhibitors with new immunotherapies such as checkpoint blockade antibodies might further enhance immune activation or counteract immunosuppressive signals.

6. Role of Immunologic Markers

A major turning point in cancer immunotherapy came with the clinical application of antibodies that block immune checkpoints. Hence, the need for clinically useful biomarkers to determine the best way to incorporate these new agents into treatment algorithms for patients with specific diseases is clear [103].

In colon cancer, T cell infiltrates in the primary tumor represent the strongest prognostic parameter compared to the currently used stage-defining parameters [98]. However, such immunological parameters have not routinely been used in clinical practice yet. In addition, determining which patients benefit from immune checkpoint inhibition remains a principal clinical question.

The importance of tumor expression of PD-L1 as a predictive biomarker has been studied extensively, and while tumor expression of PD-L1 can effectively enrich cohorts of patients, it is not a binary predictive marker [104]. Although currently one commercially available PD-L1 antibody (clone E1L3N) has been validated for IHC, the utilization of this antibody for predicting response to anti-PD-1 or anti-PD-L1 therapies remains unknown [105]. Emerging data suggest that patients whose tumors overexpress PD-L1 by IHC have improved clinical outcomes with anti-PD-1-directed therapy, but the presence of patients with PD-L1 negative tumors that also show a robust response complicates the issue of PD-L1 as an exclusionary predictive biomarker [104]. The use of PD-L1 IHC as a predictive marker is confounded by multiple unresolved issues including variable detection antibodies, differing IHC cutoffs, tissue preparation, processing variability, primary versus metastatic biopsies, oncogenic versus induced PD-L1 expression, and staining of tumor versus immune cells [106]. The utility of measuring other inhibitory components of the PD-1/PD-L1 axis such as PD-1 and PD-L2 or the role of immunostimulatory molecules like OX40 is still poorly understood. It is clear that much more information must be gathered not only on the PD-1/PD-L1 axis but also on TILs and other inhibitory/stimulatory pathways to fully understand responses and primary or acquired resistance to immunotherapy. In conclusion, a multitude of questions remain unanswered and need to be resolved to integrate predictive markers for anti-PD-1/anti-PD-L1 therapies into the clinical diagnostic routine [103, 105].

7. Discussion

The FDA approval of anti-CTLA-4 for the treatment of metastatic melanoma and of anti-PD-1 for metastatic melanoma and non-small cell lung cancer has engendered new-found awareness among oncologists of the potential antitumor activity of immune checkpoint modulation. In addition, remarkable efficacy of these drugs was shown in renal cell cancer, ovarian cancer, and Hodgkin's lymphoma, even upon failure to several lines of therapy. Despite clinical successes in a diverse range of malignancies, evidences of durable responses in CRC are scarce and appear restricted to MMR-deficient CRC, with its high mutational burden.

In CRC, due to its complicated and close relationship between the stroma and tumor cells, the combination of two or more therapeutic agents might be more effective than merely targeting a single factor. In this regard, abolishing the suppressive factors in the tumor microenvironment is only one step in this cancer-immunity cycle and still requires elimination of cancer by activated T cells. Therefore, another interesting approach could be to not only overcome immunosuppression but also combine this with agonistic antibodies such as GITR, CD27, CD40, 4-1BB, or OX40 to achieve maximum activity of the antitumor response. On the other hand, also the combination of immunotherapy with targeted therapeutics, such as the synergism between 4-1BB agonistic antibodies and Cetuximab, is promising. Nevertheless, preclinical data of combination regimens in CRC is limited and still necessitates the determination of appropriate dosing and treatment schedules of these agents. Finally, well-established biomarker candidates and detection techniques need to be developed along with therapeutic strategies targeting CAFs and the other components in the tumor microenvironment in order to be able to enhance effectiveness of immune checkpoint modulation.

Disclosure

Patrick Pauwels and Vanessa Deschoolmeester are co-senior authors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Gene Expression Profile of Dendritic Cell-Tumor Cell Hybrids Determined by Microarrays and Its Implications for Cancer Immunotherapy

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Background. Dendritic cell- (DC-) tumor fusion cells stimulate effective *in vivo* antitumor responses. However, therapeutic approaches are dependent upon the coadministration of exogenous 3rd signals. The purpose of this study was to determine the mechanisms for inadequate 3rd signaling by electrofused DC-tumor cell hybrids. *Methods*. Murine melanoma cells were fused with DCs derived from C57BL/6 mice. Quantitative real-time PCR (qPCR) was used to determine relative changes in Th (T helper) 1 and Th2 cytokine gene expression. In addition, changes in gene expression of fusion cells were determined by microarray. Last, cytokine secretion by fusion cells upon inhibition of signaling pathways was analyzed by ELISA. *Results*. qPCR analyses revealed that fusion cells exhibited a downregulation of Th1 associated cytokines IL-12 and IL-15 and an upregulation of the Th2 cytokine IL-4. Microarray studies further showed that the expression of chemokines, costimulatory molecules, and matrix-metalloproteinases was deregulated in fusion cells. Lastly, inhibitor studies demonstrate that inhibition of the PI3K/Akt/mTOR signaling pathway could restore the secretion of bioactive IL-12p70 by fusion cells. *Conclusion*. Our results suggest that combining fusion cell-based vaccination with administration of inhibitors of the PI3K/Akt/mTOR signaling pathway may enhance antitumor responses in patients.

1. Introduction

Dendritic cells (DCs) have been identified as a key component in manipulating and stimulating the immune system [1]. Activated DCs are potent antigen presenting cells that express both major histocompatibility complex (MHC) class I and II molecules (Signal 1) and costimulatory molecules (Signal 2) and secrete immune modulating cytokines (Signal 3) resulting in activation of T lymphocytes [2]. Depending on the cytokine environment, DCs may elicit either a Th (T helper) 1 or Th2 CD4 T-cell response. For tumor immunotherapy, induction of a Th1 T-cell response is pivotal, and secretion of IL-12 (interleukin 12) by DCs is of critical importance for differentiation of naive T cells into Th1 cells [3]. Furthermore, IL-12 stimulates the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) from T cells and natural killer cells. In contrast, Th2 responses, associated with cytokines IL-4, IL-5, IL-6, and IL-10, suppress Th1 activity and may anergize effector T cells to tumor antigens [4].

DCs are the basis for numerous immunotherapy strategies against a variety of cancers [5]. One of these strategies involves fusing DCs with tumor cells using electrical currents

in a method called electrofusion, hence combining the antigen presenting properties of DCs with the full repertoire of antigens present within a tumor cell in order to stimulate effector T cells [6, 7]. While DC-tumor hybrids alone are insufficient to elicit significant immune responses *in vivo* and are critically dependent upon exogenously administered 3rd signal adjuvants, murine studies using DC-tumor hybrids for vaccination given concomitantly with an adjuvant third signal, such as IL-12, OX-40-, 4-1BB-monoclonal antibody, or toll-like receptor agonists, showed regression of tumor metastases after a single vaccination in several tumor types including melanoma, breast, sarcoma, and squamous cell carcinoma [8–11]. However, systemic delivery of 3rd signal along with a DC-tumor fusion vaccine is clinically problematic due to 3rd signal toxicity and/or availability [12]. Therefore, a better understanding of the mechanisms affecting the dependence of DC-tumor fusions on 3rd signal adjuvants is of paramount importance for optimizing this immunotherapeutic approach.

In this study, we show that production of the Th1 skewing cytokine IL-12 was dramatically downregulated in DC-tumor fusion cells. Microarray analyses further reveal changes in chemokine production and expression of costimulatory molecules. In addition, gene products that are involved in signaling pathways including NFκB (nuclear factor kappa-light-chain-enhancer of activated B-cells), PI3K/Akt/mTOR (phosphatidylinositol 3-kinase/Akt, protein kinase B/mammalian target of rapamycin), Wnt (wingless-related integration site), and MAPK (mitogen-activated protein kinase) were differentially expressed in fusion cells. Inhibitor studies revealed that interruption of the canonical Wnt pathway did not affect IL-12 production by DC-tumor fusion cells and that inhibition of MEK (mitogen extracellular signal-regulated kinase) only increased IL-12 production marginally. In contrast, IL-12 production could significantly be enhanced by treatment of DC-tumor hybrids with inhibitors of the PI3K and mTOR. Given the critical role of the PI3K/Akt/mTOR signaling pathway in cancer biology and the immunostimulatory effect of PI3K/Akt/mTOR inhibitors on DC-tumor hybrids, combination therapy may represent a promising and novel cancer vaccine with enhanced clinical impact.

2. Materials and Methods

2.1. Mice. Female C57BL/6 mice were purchased from Charles River Laboratories (Raleigh, NC). The mice were maintained in a specific pathogen-free environment. All mice were used at 8 to 12 weeks of age. Animals were housed in a specific pathogen-free environment at the animal facility of the Durham Veteran Affairs Medical Center. All mice used in this study were cared for in accordance with the Guide for Humane care and use of Laboratory Animals published by the National Institutes of Health. All the animal experimental protocols were approved by the Duke University Medical Center Institutional Animal Care and Use Committee.

2.2. Tumor Cell Lines. D5LacZ is a β-galactosidase expressing derivative of the B16 F10.9 melanoma cell line and has been shown to be poorly immunogenic. Its fusion parameters as well as *in vivo* characteristics have been well studied [13]. Cells were cultured in complete media (CM) composed of RPMI 1640 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5 μg/mL fungizone, 50 μg/mL gentamicin, and 5×10^{-5} M 2-mercaptoethanol (Invitrogen, Carlsbad, CA). These cells were maintained at 37°C with 5% CO_2, harvested following a short incubation period with 0.05% trypsin with EDTA, and irradiated at 100 Gy prior to use.

2.3. DC Generation. DCs were generated from femoral and tibial bone marrow cells of C57BL/6 mice. Erythrocytes were lysed with ACK lysis buffer. B- and T-lymphocytes were depleted using antibody-coated magnetic beads (Dynal Biotech, Carlsbad, CA). The DC-enriched cell fraction was then cultured in CM supplemented with 10 ng/mL GM-CSF and 10 ng/mL IL-4 (Peprotech, Rocky Hill, NJ) at a concentration of 0.5×10^6 cells/mL at 37°C with 5% CO_2. On day 6, cells were harvested, resuspended in fresh CM + GM-CSF/IL-4 media at 1×10^6 cells/mL, and incubated at 37°C with 5% CO_2 for 24 hours. Then, LPS (lipopolysaccharide, 100 ng/mL, Sigma-Aldrich, Saint Louis, MO) was added to stimulate DC maturation. After 24 hours, FACS analysis was used to confirm mature DC phenotype as previously described [14]. After 24 hours, DCs were stained intracellularly with CFSE prior to use (Molecular Probes, Eugene, OR).

2.4. Electrofusion of DCs and Tumor Cells. Irradiated tumor cells and CFSE stained DC were mixed in a 1:1 ratio and washed in prefusion media, followed by resuspension in fusion media at a concentration of 20×10^6 cells/mL. For electrofusion, the pulse generator (model ECM 2001 generator, BTX Instruments, San Diego, CA) was used. Cells were exposed to two consecutive, independent electrical currents: (1) a low voltage alternating current of 120 V/cm for 10 seconds to achieve alignment and chain formation, and (2) a high voltage direct current of 1100 V/cm for 25 microseconds to cause a reversible breakdown of cell membranes. The multinucleated hybrid cells were allowed to stand for at least 5 minutes before incubation in culture media overnight at 37°C with 5% CO_2.

2.5. Cell Sorting. To separate unfused tumor cells (T) from T-T hybrids and unfused DCs from DC-DC hybrids FACS sorting by size on forward scatter (FSC) and side scatter (SSC) was employed (data not shown). All cells larger than the unfused cell populations were considered fusion hybrids. DC-T hybrids were purified using a combination of mechanical and FACS sorting techniques, based on their plastic adherence characteristics as well as CFSE staining. Tumor cells are adherent, while DCs are nonadherent. Therefore, after electrofusion and overnight culture, the nonadherent cell population representing unfused DCs and DC-DC hybrids was discarded. FACS was then performed only on the adherent cell population containing unfused tumor cells, T-T hybrids, and DC-T hybrids. Since only DCs were stained with CFSE, FACS sorting was used to separate CFSE positive cells

from the CFSE negative populations (unfused tumor cells and T-T hybrids). All cell samples were analyzed using the FACS Aria II (BD Biosciences, San Jose, CA).

2.6. Quantitative Real-Time PCR. 24 hours after electrofusion, total RNA was isolated using the RNeasy Plus Mini Kit protocol (Qiagen, Valencia, CA). The cDNA template was synthesized from 0.5–1.0 ug of total RNA using the RT^2 First Strand Kit protocol (SABiosciences, Frederick, MD). Each template was then combined with RT^2 SYBR Green qPCR Master Mix (SA Biosciences) and aliquoted into a 96-well mouse common cytokine plate array (SA Biosciences). The PCR cycling program was 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and then 60°C for 1 minute on a Stratagene Mx3005p qPCR machine. Analysis of qPCR data was calculated using the ΔΔCt method. Quality control guidelines were followed according to the RT^2 Profiler PCR Array System manual. Briefly, all threshold values (Ct) reported as greater than 35 indicated no detectable gene expression. Genomic DNA (GDNA) contamination was detected if the GDNA control Ct value was below 35. A Reverse Transcription Control (RTC) detected impurities in the RNA sample that affect the reverse transcription of the template and was considered positive if the Ct was greater than 5. qPCR data that did not meet quality control guidelines were excluded. All samples were run in duplicate, compared for consistency, and averaged. Gene expression associated with Th1 (IFN-γ, IL-2, IL-12p40, IL-15, IL-18, and TNF-α) and Th2 (IL-4, IL-10, IL-13, and IL-25) immune responses was analyzed.

2.7. Microarray Analysis. Total RNA was isolated from tumor cells, DCs, and DC-T fusion cells using the RNeasy Plus Mini Kit protocol (Qiagen, Valencia, CA). RNA isolation for tumor and dendritic cells was done in triplicate. For the DC-T fusion cells, RNA was extracted for each of the triplicate fusion batches. Quality check was done on a Nanodrop spectrophotometer. Triplicate samples of D5lacZ tumor cells, DCs, and DC-T fusion cells were each run through a microarray chip (Affymetrix) by the Duke DNA Microarray Core Facility. Partek Genomics Suite 6.4 (Partek Inc., St. Louis, MO) was used to perform data analysis. Robust multichip analysis (RMA) normalization was done on the entire data set. Multiway ANOVA was performed and fold change was determined to select target genes that were differentially expressed between fusion cells and DCs, or fusions cells and tumors cells, respectively. Top differentially expressed genes were selected with p value cutoff of 0.01 based on ANOVA test and fold change cutoff of >5. Hierarchical clustering was performed on differentially expressed genes based on Average Linkage with Pearson's Dissimilarity. Data was also analyzed by pathway using Metacore from Genego. Microarray data was analyzed on Excel and Metacore from Genego.

2.8. ELISA. The murine IL-4 ELISA kit (eBioscience, San Diego, CA) and the murine IL-12p70 ELISA kit (BD Biosciences, San Jose, CA) were used according to the manual provided by the manufacturer. To determine cytokine secretion by DCs or DT-tumor fusion cells, 2×10^5 cells in 1 mL of AIMV media (Invitrogen, Carlsbad, CA) were incubated in the presence of 100 ng/mL of LPS for 24 hours at 37°C, 5% CO_2. Where indicated, LPS stimulation was performed in the presence of the following inhibitors (purchased from Sigma-Aldrich, Saint Louis, MO): U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) is a highly selective inhibitor of both MEK1 and MEK2 and was used at a concentration of 100 nM, JW 74 (4-[4-(4-methoxyphenyl)-5-[[[3-(4-methylphenyl)-1,2,4-oxadiazol-5-yl]methyl]thio]-4H-1,2,4-triazol-3-yl]-pyridine) an inhibitor of the canonical Wnt pathway was used at a concentration of 10 μM, rapamycin (23,27-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclohentriacontine) forms a complex with FKBP12 (FK506 binding protein 12) that binds to and inhibits mTOR which was used at 0.5 μM, and Wortmannin which inhibits the PI3K/Akt signal transduction cascade was used at 100 nM. Experiments were performed in duplicate and error bars represent the SEM (standard error of the mean).

3. Results

3.1. The Impact of DC-Tumor Fusion on Cytokine Gene Expression. In a first set of experiments, D5LacZ tumor-tumor (T-T) cell hybrids, DC-DC hybrids, and DC-T hybrids were generated by electrofusion. Fusion cells were purified by FACS and RNA isolated from hybrid cells was analyzed by quantitative real-time PCR (qPCR) for expression levels of mRNAs encoding the Th1 cytokines IFN-γ, TNF-α, IL-2, IL-12p40 (the β-subunit of bioactive IL-12p70), IL-15, and IL-18 or the Th2 cytokines IL-4, IL-10, IL-13, and IL-25. Figure 1 shows the results of our qPCR analyses. Comparison of DC-T hybrids with DC-DC fusion cells (white bars) reveals that cytokines associated with a Th1 response including IL-12p40 and IL-15 were downregulated by more than 100- and 15-fold, respectively. In contrast, the Th2 cytokine IL-4 was dramatically upregulated by 115-fold. Among all cytokines analyzed, only TNF-α and IL-12p40 exhibited higher expression levels in DC-T fusion cells when compared to T-T fusions (Figure 1, black bars).

In another series of experiments, the Th1 and Th2 cytokine expression profile of cells exposed to electrofusion was compared to unexposed cells. However, no significant changes in cytokine gene expression between tumor cells and T-T fusion cells or DCs and DC-DC fusion cells were observed (data not shown). For this reason, we focused on the comparison of gene expression levels between DC-T hybrid cells and DCs in the subsequent analyses presented in this study.

3.2. Microarray: Cytokines and Cytokine Receptors. We next sought to determine changes in the expression of genes that may negatively impact the immunologic properties of DC-T fusion cells. In order to do so, RNAs were isolated from FACS-isolated DC-T hybrids cells, DCs, or D5LacZ tumor cells, and microarray assays were performed. Consistent with our qPCR data, expression of IL12p40 and IL-15 by DC-T fusion cells was markedly downregulated (13.2- and 8-fold)

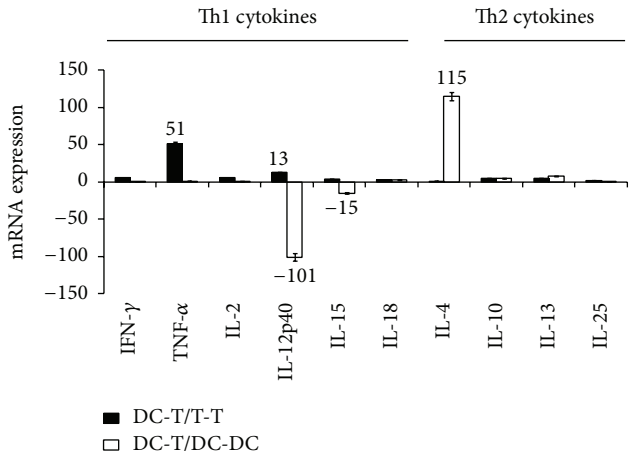


Figure 1: cDNAs generated from DC-DC hybrid cells (DC-DC), D5lacZ tumor cell hybrids (T-T), and DC-tumor cell hybrids were subjected to quantitative real-time PCR analyses using primer pairs which detect the Th1 cytokines IFN-γ, TNF-α, IL-2, IL-12p40, IL-15, and IL18 and the Th2 cytokines IL-4, IL-10, IL-13, and IL-25. Samples were run in duplicate and difference in mRNA expression was calculated using the $\Delta\Delta$Ct method. Error bars represent the standard error of the mean. One representative experiment out of three independent experiments is shown.

when compared to DCs, albeit to a lesser degree than observed in PCR analyses (Figure 2(a)). Also, IL-4 was upregulated 59.4-fold in DC-T fusions. The proinflammatory cytokines IL-1α and IL-1β were downregulated 5.5- and 8.2-fold, respectively, while TGFβ3 was upregulated 8.8-fold. Furthermore, we observed a downregulation of receptors for colony-stimulating factor (CsfR1), TNF-α (TNFR2), and IL-7 (IL-7R). In contrast the receptors for TWEAK (TNF-like weak inducer of apoptosis, TWEAKR) and for IL-17 (IL-17RC) were upregulated 9.5- and 6.5-fold. While overexpression of IL-17RC has been implicated in Bcl-2- and Bcl-X_L-independent protection of cancer cell lines from TNFα-induced apoptosis [15], TWEAKR signaling has been shown to enhance the expression of NFκB (nuclear factor kappa-light-chain-enhancer of activated B-cells)-regulated genes including IL-6, IL-8, RANTES, and ICAM-1 (CD54) [16]. However, upregulation of none of these gene products was observed in our study (Figures 2(a), 2(c), and 3(a)).

3.3. Microarray: Gene Products Involved in Cytokine Signaling. In addition to cytokine gene and cytokine receptor expression, there were also significant changes in the expression level of gene products that are involved in cytokine signaling (Figure 2(b)). Expression of TGFβi (transforming growth factor beta-induced), a protein that is induced by TGFβ and that acts to inhibit cell adhesion [17], was downregulated 14.1-fold. Downregulation of this gene product was unexpected given that TGFβ3 was upregulated in DC-T hybrids (Figure 2(a)) and implies that the TGFβ-signaling pathway may not be hyperactive in DC-T hybrid cells. There were no differences in expression levels of TGFβ-receptors between DCs and DC-T hybrid cells. However, NEDD4L (neural precursor cell expressed developmentally downregulated gene 4-like) was upregulated 13.8-fold in DC-T hybrid cells. NEDD4L negatively regulates TGFβ signaling by ubiquitination-mediated degradation of TGF-β receptor 1 and receptor-regulated Smad2 (mothers against decapentaplegic homolog 2) [18]. As such, it is reasonable to assume that NEDDL4 overexpression suppressed transcriptional activity induced by TGFβ.

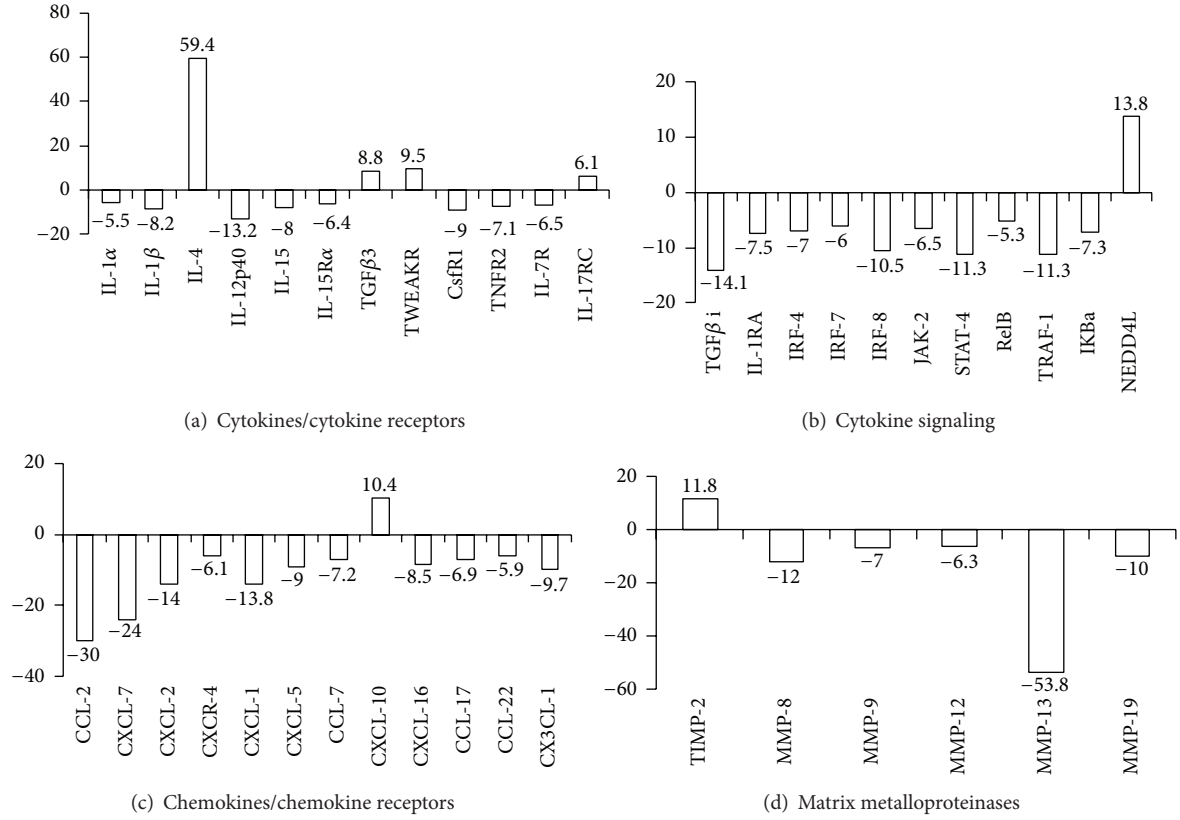
Expression of IL-1RA, the interleukin-1 receptor antagonist, which modulates a variety of IL-1 related immune and inflammatory responses, was downregulated 7.5-fold. Moreover, expression of IRFs (interferon regulatory factors) 4, 7, and 9, which are involved in transcriptional regulation of type I interferon genes, interferon signaling, and hence the Janus kinase- (JAK-) Signal Transducer and Activator of Transcription (STAT) pathway [19], was downregulated 7-, 6-, and 10.5-fold, respectively. Also, JAK-2 and STAT-4, known to be involved in IL-12 receptor signaling [20], are downregulated 6.5- and 11.3-fold.

Last, three gene products that are associated with NF-κB signaling were found to be downregulated in DC-T hybrid cells, namely, RelB (reticuloendotheliosis viral oncogene homolog B, 5.3-fold), TRAF-1 (TNF receptor associated factor-1, 11.3-fold), and the NF-κB inhibitor IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha, 7.3-fold).

RelB is known to form heterodimers with NF-κB p50 or p52 [21], and TRAF-1 forms a heterodimeric complex with TRAF2, which is required for TNF-α-mediated activation of MAPK8/JNK (Jun kinase) and NF-κB. On the other hand, the inhibitor of NF-κB [22], IκBα, is also downregulated. These results are somewhat contradictory and argue that NF-κB activity in DC-tumor fusion cells is not regulated at the transcriptional level.

3.4. Microarray Analyses: Chemokines and Chemokine Receptors. As shown in Figure 2(c), expression of chemokines or their receptors which are involved in chemotaxis of neutrophils, monocytes, DCs, T cells, and NK cells were generally downregulated in DC-tumor fusions, with the exception of CXCL-10 (IP-10, interferon-gamma-induced protein 10). Surprisingly, even chemokines involved in chemotaxis of Th2 cells and regulatory T cells (CCL-17 and CCL-22) were downregulated while IP-10 which is implicated in the induction of Th1 responses and chemotaxis of Th1 cells was significantly upregulated [23, 24]. We therefore hypothesize that the chemokine expression profile of DC-tumor hybrids does not have a major impact on the Th-polarizing capacity of DC-tumor hybrid cells.

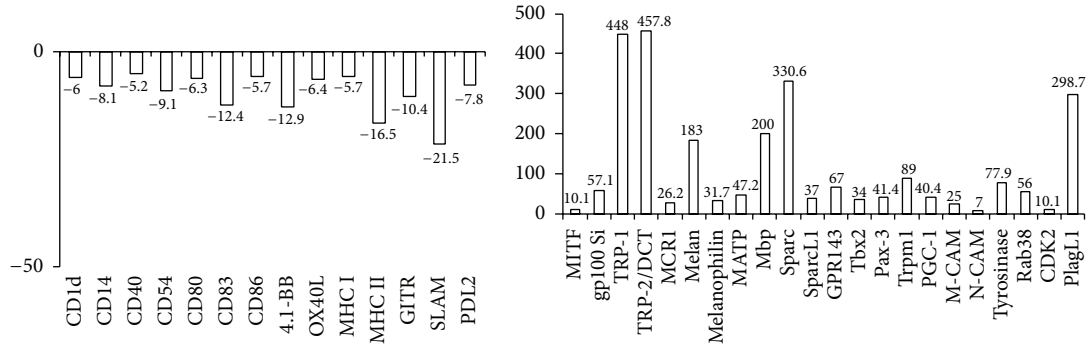
3.5. Microarray Analyses: Matrix Metalloproteinases (MMPs). It has been demonstrated that the expression of matrix metalloproteinases MT-1 (MMP-14) and MMP-9 is a major contributing factor to the migratory capacity of DCs to lymph nodes through the degradation of extracellular matrix components. In this context, MMP-9 activity is of particular importance since it cleaves collagen IV, a major component of basement membranes. Furthermore, it has been shown that the balance of MMP-9 and TIMP (tissue inhibitor of MMPs) expression is crucial for DC migration *in vivo* [25].



(a) Cytokines/cytokine receptors (b) Cytokine signaling

(c) Chemokines/chemokine receptors (d) Matrix metalloproteinases

Figure 2: Expression profile of cytokines and cytokine receptors. (a) Molecules involved in cytokine signaling, (b) chemokines and chemokine receptors, and (c) matrix metalloproteinases (d). Data from microarrays are presented and fold differences in expression between DC-tumor cell hybrids and DCs are shown. Positive values indicate upregulation in DC-tumor cell hybrids, while negative values indicate downregulation. When expression levels from different probe sets were available for the same gene product (IL-12p40, CsfR1, TGFβi, IL-1RA, and TIMP-2), the average of their values was presented.



(a) Costimulation/inhibition and Ag presentation (b) Melanoma-associated gene products

Figure 3: Expression level of costimulatory molecules and molecules involved in antigen (Ag) presentation (a) or melanoma-associated gene products (b) in DC-tumor cell hybrids. Microarray data for DC-tumor cell hybrids and DCs were compared. Positive values indicate upregulation in DC-tumor cell hybrids. Data from different probe sets were obtained for CD1d, HLA-class II (HLA-Q6 and HLA-Q7), MITF, Melanophilin, Pax3, PGC-1, MATF, SPARC, Mbp, Tyrosinase, Trp1, and Trp2. For these gene products the average in gene expression is shown.

Our data reveal that TIMP-2 was upregulated 11.8-fold in DC-T fusion cells, while MMP-9 is downregulated 7-fold (Figure 2(d)). As such, these results suggest that the migratory capacity of DC-T hybrids toward lymph-node derived chemokines, namely, CCL-19 and CCL-21, may be impaired.

3.6. Microarray Analyses: Costimulatory Molecules and Antigen Presentation. As shown in Figure 3(a), expression of genes involved in antigen presentation in the context of MHC classes I and II or Cd1d was downregulated 5.7-, 16.5-, and 6-fold in fusion cells. Furthermore, the expression of all well-established costimulatory molecules, including CD40,

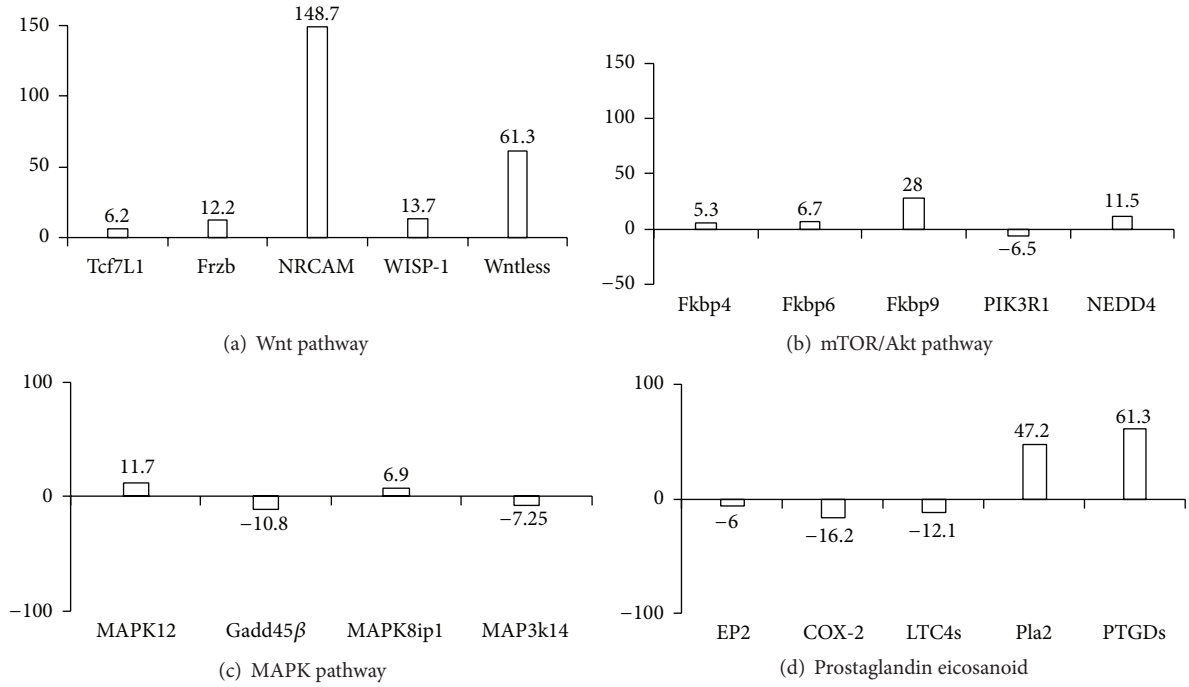


Figure 4: Expression level of molecules involved in signal transduction of the Wnt pathway (a), the mTOR/Akt pathway (b), the MAPK pathway (c), or prostaglandin, eicosanoid pathway (d). Microarray data for DC-tumor cell hybrids and DCs were compared. Positive values indicate upregulation in DC-tumor cell hybrids. Data from different probe sets were obtained for Frzb, WISP-1, and Gadd45β. For these gene products the average in gene expression is shown.

CD54, CD80, CD83, CD86, 4-1BB, GITR (glucocorticoid-induced TNFR-related protein), OX40L, and SLAM (signaling lymphocytic activation molecule), was downregulated in DC-tumor fusion cells. These data explain to some degree why targeting of costimulatory molecules with agonistic antibodies can enhance the potency of DC-tumor fusion-based vaccines, as has been described previously.

Last, expression of PD-L2 (programmed death ligand 2), an inhibitory immune checkpoint molecule, was suppressed 7.8-fold in DC-fusion cells. No differences in PD-L1 expression between DCs and DC-T hybrid cells were observed.

3.6.1. Microarray Analyses: Melanoma-Associated Gene Products. The development of melanocytes is highly dependent on the action of the microphthalmia-associated transcription factor (MITF) which has been shown to regulate a broad variety of genes, whose functions range from pigment production to cell-cycle regulation, migration, and survival [26]. MITF was upregulated in DC-tumor fusion cells (Figure 3(b)). Concomitantly, also MITF-regulated mRNAs encoding melanoma antigens, including Tyr (Tyrosinase), TRP-1 and TRP-2 (Tyrosinase-related protein), gp100 (Silver), Melan, Melanophilin, M-CAM (melanoma cell adhesion molecule), and MATP (membrane-associated transporter protein also known as solute carrier family 45 member 2 (SLC45A2) or melanoma antigen AIM1), were also highly upregulated. Moreover, expression of MITF-regulated MCR1 (melanocortin 1 receptor), TRPM1 (transient receptor potential cation channel subfamily M member 1), GPR143 (G protein-coupled receptor 143), and Mbp (myelin basic protein) was highly upregulated in fusion cells. Expression of Mbp by melanoma cells is somewhat surprising, but it has been shown that B16F10 cells undergo differentiation to a myelinating glial phenotype characterized by induction of the transcriptional activity of the MBP promoter [27].

Last, Osteonectin (secreted protein acidic and rich in cysteine (SPARC)), which has been implicated in metastasis of melanoma to the lungs [28], and PlagL1 (Pleomorphic adenoma gene-like 1), a potential tumor suppressor gene [29], were also overexpressed in DC-tumor fusions. These results suggest that the entire antigenic repertoire of melanoma cells is indeed strongly expressed in DC-tumor hybrid cells, as has been hypothesized.

3.6.2. Microarray Analyses: Signal Transduction Pathways. Next, we analyzed expression levels of genes that are involved in signaling pathways known to be aberrantly regulated in cancer cells [30]. The transcription factor Tcf7L1 (transcription factor 7-like 1) which is activated by β-catenin and thus mediated Wnt signaling was upregulated 6.2-fold (Figure 4(a)). Also, expression of Frzb (Frisbee), a Wnt-binding protein and competitor for the cell-surface receptor Frizzled, and expression of Wntless (G protein-coupled receptor 177), another receptor for Wnt proteins, were increased 12.2- and 61.3-fold. Furthermore, target genes of the canonical Wnt pathway, WISP-1 (WNT1-inducible-signaling pathway protein 1) [31] and NRCAM (neuronal cell adhesion molecule) [32], were upregulated 13.7- and 148.7-fold, indicating activation of the Wnt pathway in DC-tumor fusion cells.

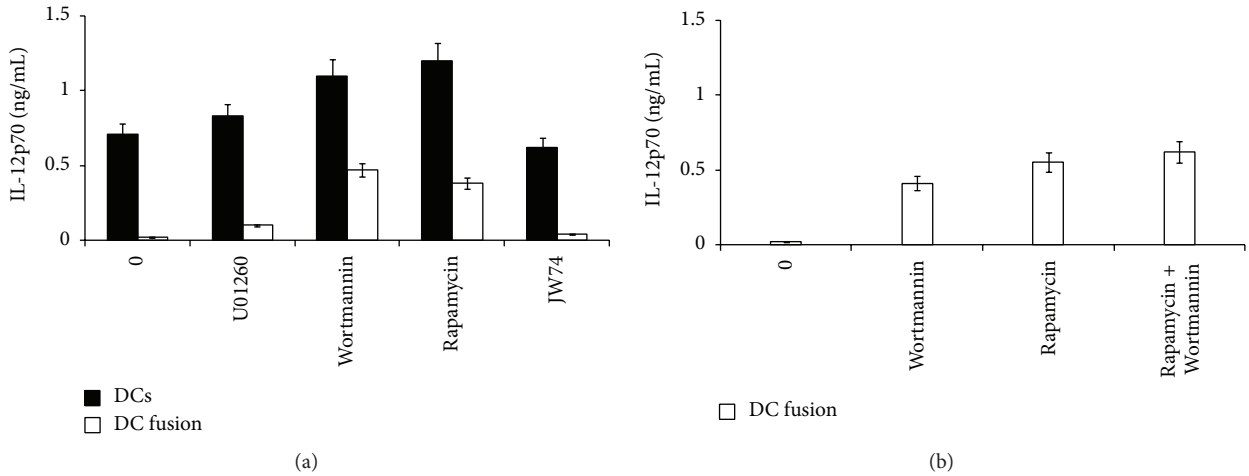


Figure 5: (a) Secretion of IL-12p70 by DCs and DC-tumor cell hybrids in the presence of signal transduction inhibitors. DCs (black bars) or DC-tumor cell hybrids (white bars) were stimulated with LPS and supernatants were analyzed by ELISA as described in Section 2. Stimulations were performed in the absence of inhibitor (0), or in the presence of MEK inhibitor (U0126), PI3K inhibitor Wortmannin, mTOR inhibitor rapamycin, or JW74, an inhibitor of the canonical Wnt pathway. Experiments were performed in duplicate and the standard error of the mean is presented. (b) Secretion of IL-12p70 DC-tumor cell hybrids. DC-tumor cell hybrids were stimulated with LPS in the presence or absence of inhibitors as indicated. Experiments were performed in duplicate and the standard error of the mean is presented.

Expression of the FK506 binding proteins FKBP4, FKBP6, and FKBP9, immunophilins known to interact with mTOR [33], was upregulated in DC-tumor fusions 5.3-, 6.7-, and 28-fold (Figure 4(b)). Furthermore, expression of PIK3R1 (phosphatidylinositol 3-kinase regulatory subunit alpha, p85α) was downregulated 6.5-fold, which may indicate aberrant activity of PI3K in DC-tumor fusion cells. In addition, we observed that NEDD4 was upregulated 11.5-fold. NEDD4 directly binds to and poly-ubiquitinates PTEN (phosphatase and tensin homolog), targeting it for proteasomal degradation [18]. PTEN is a tumor suppressor that negatively regulates the PI3K/Akt pathway. Therefore, posttranslational suppression of its expression level may lead to hyperactivation of the PI3K/Akt signaling pathway [18].

The LPS-inducible mitogen-activated protein kinase 12 (MAPK12), also known as extracellular signal-regulated kinase 6 (ERK6) or p38-γ, was upregulated 11.7-fold in DC-tumor fusions (Figure 4(c)). In contrast, the LPS-inducible Gadd45β (growth arrest and DNA damage-inducible 45) was downregulated 10.8-fold. Gadd45β is an NF-κB target gene which, in combination with MEKK4, activates p38MAPK [34]. Furthermore, mitogen-activated protein kinase kinase kinase 14 (MAP3K14) also known as NF-kappa-B-inducing kinase was downregulated 7.25-fold in fusion cells. This kinase is known to bind to TRAF2 and to stimulate NF-κB activity [35]. Lastly, expression of C-jun amino-terminal kinase interacting protein 1 (MAPK8ip1), a negative regulator of MAPK8 (c-jun amino-terminal kinase) [36], was upregulated 6.9-fold in DC-tumor hybrid cells.

Lipid mediators such as prostaglandins have been implicated in tumor-mediated immunosuppression [37, 38]. As presented in Figure 4(d), several genes involved in eicosanoid biosynthesis and signaling were differentially expressed in DC-tumor fusion cells. The prostaglandin E2 receptor 2 (EP2) was downregulated 6-fold. Additionally, cyclooxygenase-2 was downregulated 16.2-fold and the cysteinyl-leukotriene C4 synthase (LTC4s) was downregulated 12.1-fold. In contrast, phospholipase A2 (Pla2) and prostaglandin D2 synthase (PGDs) were upregulated 47.2- and 61.3-fold, respectively.

3.7. Inhibitor Studies. We next sought to determine whether inhibition of signaling pathways, for which inhibitors are available clinically, could restore secretion of bioactive IL-12p70 by DC-tumor fusion cells. We chose Wortmannin as an inhibitor of PI3K upstream of Akt (PKB), U0126 as an inhibitor of MEK1 and MEK2, rapamycin as an inhibitor of mTOR, and JW74 as an inhibitor of the canonical Wnt pathway. Admittedly, our data provide several lines of evidence that NF-κB-signaling is impaired in DC-tumor fusion cells, but, even though NF-κB-inhibitors are starting to emerge in the clinic, it would obviously not make sense to administer NF-κB-agonists to cancer patients. We therefore omitted stimulators of NF-κB-activity in our assay.

DCs and DC-tumor fusion cells were stimulated with LPS in the presence or absence of inhibitors as indicated in Figure 5(a) and supernatants were analyzed for IL-12p70 secretion by ELISA. As expected, DC-tumor fusions did not produce IL-12p70 in response, while DCs responded to LPS stimulation. U0126 led to a modest increase of IL-12p70 by both DC-tumor fusions and DCs. Inhibition of PI3K with Wortmannin and inhibition of mTOR with rapamycin increased secretion of IL12-p70 significantly (11-13-fold). Inhibition of the canonical Wnt pathway with JW74 did not have any impact on IL-12p70 production by DCs or DC-tumor fusion cells. We next asked whether combined inhibition of PI3K and of mTOR could further enhance IL-12p70 secretion by DC-tumor fusion cells. As shown in Figure 5(b), combining Wortmannin and

rapamycin to inhibit PI3K and mTOR did not significantly enhance IL-12p70 secretion by DC-tumor hybrid cells, hence excluding a synergistic or additive effect of these inhibitors.

4. Discussion

This study is the first to investigate the mechanisms responsible for the dependence of DC-tumor hybrid vaccines on exogenously provided 3rd signal adjuvants. Several hypotheses have been postulated regarding tumor cell-mediated inhibition of immune responses. These include the induction of apoptosis of immune cells via expression of Fas ligand, TRAIL (TNF-related apoptosis-inducing ligand) [39, 40], or PD-L1 and PD-L2 (programmed death ligand) [41]. Furthermore, induction of tolerance through cytokines such as TGF-β, IL-6, and IL-10 [42] or lipid mediators [37, 38] has been described. Lastly, activation of the MAPK pathway by melanoma cells has been described as a mechanism to inhibit IL-12 production by DCs in a paracrine manner [43]. Our results do not provide evidence for overexpression of apoptosis-inducing ligands by DC-tumor cell hybrids, nor did we observe an enhanced production of tolerance-inducing cytokine IL-6 or IL-10 by these cells. TGF-β3 was upregulated 8.8-fold in DC-tumor fusions, but the observed downregulation of the TGFβ-induced protein in combination with upregulation of NEDD4L argues against a major impact of this cytokine on fusion cells.

Surprisingly, despite a profound upregulation of mRNA encoding IL-4 in DC-tumor hybrids, there was no evidence of IL-4 signaling in these cells. We did not observe any upregulation of target genes of the IL-4 receptor I or II, including SOCS-1, IL-4 receptor α, CCL11 (eotaxin 1), or Fcε receptor II. In addition, there were no changes in expression of gene products that are components of the IL-4 receptors, namely, IL-4 receptor α, IL13 receptor α, and CD25.

Even though the expression levels of phospholipases A2, which release arachidonic acid from phospholipids, and of prostaglandin D2 synthase were upregulated in DC-tumor fusions, expression of cyclooxygenase, which catalyzes the downstream conversion of arachidonic acid into eicosanoids, was downregulated. Furthermore, while PGD-2 has been shown to upregulate CD80 and to downregulate IP-10 in LPS-matured DCs [44], the exact opposite was observed in our experiments (Figures 2(c) and 3(a)). Accordingly, we conclude that PGD-2 may not be the main culprit for the dramatic downregulation of IL-12 production in DC-tumor hybrids.

Dysregulation of the MAPK pathway in melanoma cells has been extensively investigated [45]. However, it has been described that, in the spontaneous B16F10 melanoma cell line, expression of p16Ink4a (inhibitor of CDK4a), which inhibits cell-cycle progression by inactivating cyclin-dependent kinases, and of p19Arf (alternate reading frame tumor suppressor), which causes Mdm2 (mouse double minute 2 homologue) induced translational silencing and p53 degradation, is lost and that there is no evidence of activation of the MAPK-signaling pathway in this cell line [46]. It is therefore highly unlikely that the MAPK-signaling pathway would be a major contributor to the loss of immunostimulatory capacity of DC-tumor hybrid cells. Nevertheless, our data reveal that treatment of DC-tumor hybrid cells with MEK inhibitor U0126 led to a modest increase in IL-12 secretion. This however might be a result of the previously published observation that treatment with U0126 can result in a slight but significant inhibition of $p70^{S6K}$ (S6 ribosomal protein kinase) activation, a downstream target of Akt [47].

Our results further indicate that molecules that are involved in Wnt signaling, including Tcf7L1, Frzb, and Wntless, were upregulated in DC-tumor fusions. Additionally, WISP-1 and NRCAM, targets of the canonical Wnt pathway, were upregulated 12- and 148.7-fold, respectively. In the canonical Wnt pathway, activation of Wnt receptors leads to stabilization and import of β-catenin into the nucleus where β-catenin associates with T-cell factor/lymphoid enhancer factor (TCF/LEF) and activates target genes. However, treatment of DC-tumor fusions with JW74, a specific inhibitor of the canonical Wnt pathway, had no impact on IL-12 secretion by these cells. On the other hand, it is conceivable that the mTOR pathway was activated through the noncanonical Wnt/Ca^{2+} pathway, Wnt-dependent activation of PKA (protein kinase A) and CREB (cAMP response element-binding protein), or mTOR activation via Wnt-mediated inhibition of glycogen synthase kinase 3. Alternatively, we cannot exclude that the PI3K/Akt/mTOR pathway was activated independent of Wnt signaling.

The PI3K/Akt/mTOR pathway has been shown to play a critical role in cell proliferation, survival, and metastasis of cancer cells [48], and we observed that inhibition of the PI3K/Akt *and inhibition* of the mTOR pathway enhanced the immune-stimulatory capacity of DC-tumor fusions through induction of bioactive IL-12p70 secretion. The fact that combined inhibition of PI3K and mTOR signaling did not further improve IL-12p70 secretion by DC-tumor fusions may indicate that inhibition acted on the same signaling pathway, likely to involve $p70^{S6K}$ as has been described previously [49].

In sum, we conclude that combining PI3K/Akt/mTOR inhibition with DC-melanoma fusion cell-based cancer vaccination appears to be a promising strategy and warrants further studies *in vitro* and in animal models. Ultimately, this research may lead to the development of improved DC-fusion-based cancer vaccines with enhanced clinical impact.

Abbreviations

Akt/PKB:	Protein kinase B
CFSE:	Carboxyfluorescein diacetate succinimidyl ester
CM:	Complete media
DC:	Dendritic cell
ELISA:	Enzyme-linked immunosorbent assay
FACS:	Fluorescence-assisted cell sorting
IFN:	Interferon
IL:	Interleukin
MEK:	Mitogen extracellular signal-regulated kinase
mTOR:	Mammalian target of rapamycin
LPS:	Lipopolysaccharide

PI3K: Phosphatidylinositol 3-kinase
qPCR: Quantitative real-time PCR
Th: T helper.

Disclaimer

The views expressed in this paper are those of the authors and do not necessarily represent the views of the Department of Veterans Affairs or the United States government.

Conflict of Interests

The authors have no competing interests.

Authors' Contribution

Jens Dannull and Chunrui Tan equally contributed by performing experiments, data analysis, and drafting the paper. Christine Farrell and Cynthia Wang performed experiments and data analysis and helped in editing the paper. Scott Pruitt and Smita K. Nair provided input on experimental design, data analysis, and paper revisions. Walter T. Lee was responsible for experimental design, experimental supervision, data analysis, paper drafting, and review. Jens Dannull and Chunrui Tan contributed equally to this study.

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Review Article

Cellular and Antibody Based Approaches for Pediatric Cancer Immunotherapy

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Progress in the use of traditional chemotherapy and radiation-based strategies for the treatment of pediatric malignancies has plateaued in the past decade, particularly for patients with relapsing or therapy refractory disease. As a result, cellular and humoral immunotherapy approaches have been investigated for several childhood cancers. Several monoclonal antibodies are now FDA approved and commercially available, some of which are currently considered standard of practice. There are also several new cellular immunotherapy approaches under investigation, including chimeric antigen receptor (CAR) modified T cells, cancer vaccines and adjuvants, and natural killer (NK) cell therapies. In this review, we will discuss previous studies on pediatric cancer immunotherapy and new approaches that are currently being investigated in clinical trials.

1. Introduction

Each year there are an estimated 15,780 children (age less than 19 years) who are diagnosed with cancer in the United States [1] and approximately 250,000 children worldwide [2]. While use of chemotherapy and radiation approaches has resulted in improved cure rates, cancer remains the most common cause of disease-related mortality in America. Children with relapsing or therapy refractory cancer have limited treatment options with further intensification of chemotherapy or radiation. With the additive toxicities of conventional treatment approaches and limited efficacy in achieving cure, many pediatric immunotherapy studies have targeted patients with relapsing cancer in a Phase I setting, with a long range goal of using immune-based therapy to prevent relapse or treat minimal disease.

Ongoing challenges in pediatric cancer immunotherapy include identifying subjects who may be able to benefit from this approach, since many of these patients have significant immunocompromise from previous therapy, and have limited ability to achieve an immune response to target antigens. For this reason, there has been much interest in the use of adjuvant agents in the setting of cancer vaccines, adoptive cellular immunotherapy, and the use of monoclonal antibodies. Advances in technology over the past decade have resulted in increased understanding of cancers on a genomic level as well as identification of new tumor-associated antigens. This in turn has paved the way for the development of novel monoclonal antibody and cell-based immunotherapy agents. In this review, we will discuss immunotherapy with monoclonal antibodies (mAbs), dendritic cell (DC), and cancer vaccines, as well as cellular immunotherapy with NK cells, CAR T cells, and antigen specific cytotoxic T lymphocytes (CTL).

2. Monoclonal Antibodies

mAbs work by binding to antigens on the tumor cell surface and either facilitating antibody-dependent cellular cytotoxicity (ADCC) by the host's immune system or more directly serving as a vector for a toxin or radionuclide (Figure 1). The main advantage of mAbs over cell-based approaches (e.g., CAR and tumor vaccines) is that they can be stored in clinic and hospital pharmacies and advanced expertise in cell-based therapeutics is not needed.

Rituximab is a mAb targeting CD20, an antigen expressed on B-cell lymphomas, and became the first ever mAb approved for clinical use in 1997. It is approved for use in

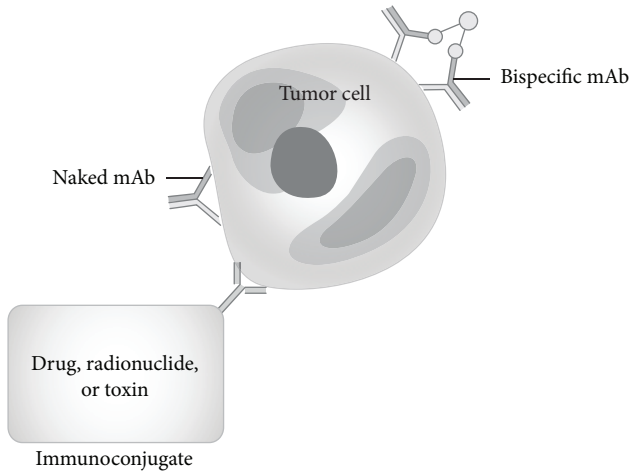

Figure 1: Different mechanisms of tumor cell killing by monoclonal antibody therapy. Monoclonal antibodies exhibit tumor cell cytotoxicity by targeting a specific tumor antigen. Immunoconjugates are monoclonal antibodies conjugated to drugs, toxins (immunotoxins), or radionuclides. mAb: monoclonal antibody.

non-Hodgkin lymphoma (NHL) as well as chronic lymphocytic leukemia. CD20 is present in virtually all patients with lymphocyte predominant Hodgkin lymphoma (LPHL) and in a significant minority of patients with classical Hodgkin lymphoma (HL). In one Phase II trial for LPHL, rituximab showed a 96% overall response rate, with 75% 1-year EFS [3]. This antibody has also been used successfully to treat B-cell lymphoproliferative disease and lymphomas following solid organ and stem cell transplantation [4]. While the use of anti-B-cell therapy often results in hypogammaglobulinemia, this is deemed relatively safe given the availability of gamma globulin replacement.

In 2011, brentuximab vedotin, an anti-CD30 mAb conjugated to monomethyl auristatin E, a microtubule inhibitor, was approved by the FDA for relapsing or refractory HL and anaplastic large cell lymphoma (ALCL). Overall response rates in several case reports of pediatric relapsing HL or ALCL showed a 47–64% overall response rate [5]. A Children's Oncology Group (COG) study is underway looking at administering brentuximab vedotin and both eliminating bleomycin (due to potential risk of increased pulmonary toxicity with concurrent use) and decreasing the cumulative dose of vincristine, another antimicrotubule agent.

In 2000, the FDA approved gemtuzumab ozogamicin (GO) for acute myelogenous leukemia (AML), an anti-CD33 mAb conjugated to the drug calicheamicin. The drug was later withdrawn from the market in 2010 due to concerns of hepatic sinusoidal obstruction syndrome and lack of statistically significant clinical benefit in an adult Phase III trial [6]. Subsequent studies have shown that lower doses of GO than previously used could be safely administered, leading to renewed interest in clinical studies with this agent [7]. Inotuzumab (CMC-544) is an anti-CD22 conjugate linked to ozogamicin which has shown activity in Phase II trials in pediatric B-cell ALL [8]. Studies are underway to better elucidate its role in refractory or relapsing pediatric B-ALL. Moxetumomab pasudotox is an anti-CD22 mAb conjugated to a pseudomonal exotoxin that is being investigated in pediatric B-cell ALL prior to allogeneic stem cell transplantation (SCT). An anti-CD22/anti-CD19 mAb agent is also undergoing clinical investigation and has shown promising results in a Phase I study for refractory or relapsing pediatric B-cell ALL [9]. mAbs conjugated to radionuclides including CHT-25 (anti-CD25 mAb conjugated to 131-iodine) and ibritumomab (anti-CD20 mAb conjugated to 90-yttrium) have shown efficacy in pediatric Hodgkin and non-Hodgkin lymphoma, respectively [10, 11]. Radionuclide immunoconjugates, however, can lead to prolonged cytopenias, limiting their use.

A new class of mAbs, called bispecific antibodies, are molecules that recognize two distinct antigens on the tumor cell surface. Blinatumomab is a bispecific T-cell engager (BiTE) that targets CD19 positive cells and simultaneously binds to CD3-positive, activated T cells for killing. This mechanism of action allows bypassing MHC Class I restriction to achieve killing by T cells. A COG Phase II study incorporating its use in both ALL and B-cell lymphoma is underway. There is also preclinical data supporting the possibility of targeting T cells against neuroblastoma with the use of 3F8BiAb, a bispecific antibody to GD2 (present on neuroblastoma cells) and CD3 (expressed on activated T cells) [12].

Monoclonal antibodies targeting the disialoganglioside GD2, which is expressed on tumors of neuroectodermal origin, have been in clinical trials for over two decades. GD2 is an ideal target for neuroblastoma since its expression is highly restricted on normal tissues, principally to the cerebellum and peripheral nerves. The chimeric mAb ch14.18 against GD2 has become the most widely used mAb in pediatric cancer and its use in the adjuvant setting following standard neuroblastoma therapy has resulted in improved survival for patients with metastatic disease. A pivotal study was performed by the COG in which there was an improved 2-year EFS of 64% (compared to 44% with cis-retinoic acid alone) when given with aldesleukin (IL-2) and granulocyte monocyte colony stimulating factor (GM-CSF) [13]. Immunotherapy with anti-GD2 has now become the standard of care for patients with metastatic neuroblastoma. Another anti-GD2 drug, humanized 14.18-3F8 conjugated to IL-2, has shown activity in Phase II trials in children with refractory/relapsing neuroblastoma [14]. GD2 is also highly expressed on osteosarcomas [15], and Phase I studies are underway to investigate its role in the therapy of this tumor.

Tumor signaling and growth pathways have also served as target antigens in pediatric solid tumors. Vascular Endothelial Growth Factor (VEGF) is a signaling protein that is critical for solid tumor vascular proliferation. Bevacizumab, a VEGF inhibitor, has shown activity both as a single agent and in combination with other chemotherapy agents for a variety of tumors, including recurrent low grade glioma [16, 17], medulloblastoma [17, 18], neuroblastoma, rhabdomyosarcoma, Wilms tumor, and hepatocellular carcinoma [19]. Cetuximab is a mAb directed against Epidermal Growth Factor (EGF) receptor and although it is approved for several adult malignancies, its role and potential benefit in pediatric solid tumors are still being investigated. Human Epidermal

Growth Factor Receptor 2 (HER2) expression, which is typically considered a biomarker for breast cancer, has been associated with poor outcome in osteosarcoma. The use of trastuzumab, an anti-HER2 mAb, did not result in significant differences in EFS and OS when studied in conjunction with standard chemotherapy in patients with metastatic osteosarcoma [20]. The Insulin Growth Factor-1 receptor (IGF-1R) pathway has been the target of various mAbs, but clinical efficacy has been variable. The administration of figitumumab, an anti-IGF-1R mAb, has been associated with objective responses in Ewing sarcoma patients [21], whereas R1507, another IGF-1R antagonist mAb, had mixed results in two studies [22, 23]. Racotumomab, an anti-idiotype vaccine targeting NeuGcGM3, when tested in children (Phase I study) with relapsing or resistant neuroblastoma and other tumors expressing NeuGcGM3, showed IgM and IgG response in most patients [24].

Monoclonal antibodies that target the T-cell inhibitory checkpoints are also undergoing investigation for pediatric solid tumors. Programmed cell death receptor (PD1) is a cell surface receptor that plays an important role in downregulating T-cell activation, which in turn leads to tumor tolerance. Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) is another protein receptor that functions as an immune checkpoint and helps downregulate the immune system. Nivolumab, an anti-PD1 mAb, and ipilimumab, an anti-CTLA-4 mAb, are FDA approved for the treatment of melanoma. Nivolumab with or without ipilimumab is being investigated for the treatment of recurrent or refractory pediatric solid tumors.

3. Chimeric Antigen Receptor T Cells

Recent advances in cell culture and manipulation technology have resulted in the ability to expand clinically relevant numbers of engineered T cells that express chimeric antigen receptors (CARs). CARs are genetically engineered receptors that redirect T cells to a selected tumor antigen (Figure 2). Cancer cells often escape T-cell immune surveillance by downregulating HLA molecules involved in antigen presentation. The main advantage of CAR T cells is that this approach bypasses the need for tumor antigen presentation to MHC Class I molecules, hence providing the dual benefit of target specificity akin to mAbs and the killing capacity of CTL. Autologous T cells are collected from the patient and subsequently expanded; CARs are then genetically inserted into those T cells using viral vectors, DNA transposons, or RNA transfection [25]. These CAR T cells can later be reinfused to the patient to treat refractory malignancies. First-generation CARs consist of a single Fv fragment or activation domain against a tumor-associated antigen. Second- and third-generation CAR T cells involve the addition of one or two costimulatory molecules (e.g., CD28 and CD137), respectively. The addition of a costimulatory molecule in second-generation CAR T cells has led to demonstrable improvement in T-cell proliferation and survival [26].

There are at least 30 studies on https://clinicaltrials.gov/ involving CAR T cells that allowed for pediatric enrollment. Less than a third were designed for solid malignancies, with

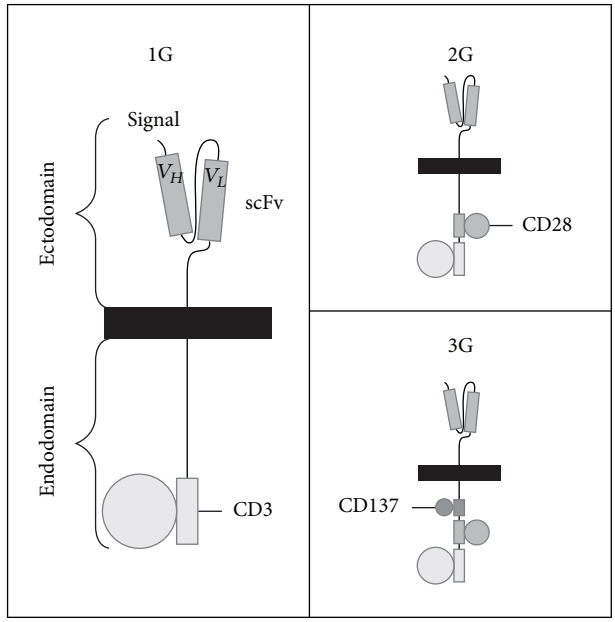

Figure 2: Different generations of chimeric antigen receptors (CARs). Left to right, top to bottom. First-generation CARs consist of scFv fragment against a tumor antigen (e.g., GD2 in neuroblastoma, CD19 in B-cell malignancies) linking a CD3 signaling chain. Second- and third-generation CARs incorporate 1 or 2 costimulatory molecules, respectively (e.g., CD28, CD137). 1G: first generation; 2G: second generation; 3G: third generation.

the majority aimed at CD19+ hematologic malignancies. Sustained complete remissions were attained in adults with B-cell ALL, NHL, and refractory CLL by targeting CD19 [27–29]. Based on the dramatic responses noted in adults, CD19 CAR T-cell infusions were performed in pediatric ALL with similar outcomes. In the first Phase I trial, 2 children with refractory, heavily pretreated B-cell ALL achieved complete remissions, with one relapsing from CD19-negative disease 2 months after CD19 CAR T-cell therapy [30]. A study from Children's Hospital of Philadelphia reported outcomes in 25 children receiving CAR T cells, the majority of whom received a prior allogeneic SCT. Complete remission was achieved in up to 90% of patients, which included 2 patients previously treated with blinatumomab. Event-free and overall survival at 6 months was 67% and 78%, respectively, and durable remissions up to 24 months were observed [31]. CARs targeting other lymphoid (e.g., CD22, CD30) and myeloid (e.g., CD13, CD33) antigens are currently in development. B-cell aplasia leading to prolonged hypogammaglobulinemia is a concern with CD19-targeted CAR T-cell therapy. In one study which looked at CD19-targeted CAR T-cell therapy in 21 children and young adults (age <30 years), no cases of prolonged B-cell aplasia were observed [32]. Trials looking at targeting CD30 and CD33 for other hematologic malignancies are ongoing.

There is limited data with use of CAR T cells for malignant solid tumors. In a Phase I study using first-gen-eration CAR T cells targeting GD2 for refractory neuroblastoma, 27% of those with active disease at time of

the anti-GD2 CAR T-cell infusion subsequently went into complete remission, with 2 durable remissions >60 months [33]. Another first-generation CAR T-cell study targeting tumor-associated CD171, an L1 cell adhesion molecule, did not show clinical efficacy. CAR T cells are being included in clinical trials targeting IL-13R alpha, which is expressed on gliomas and medulloblastomas not on normal CNS tissue, GD2 for osteosarcoma, and HER2, which is highly expressed in osteosarcoma and some cases of medulloblastoma.

Despite success with CAR T cells, there are significant potential toxicities associated with this therapy. Cytokine release syndrome is a known side effect from the CAR T-cell infusion but is reversible with the benefit of tocilizumab, an anti-inflammatory agent against IL-6 [34]. Severe, life-threatening cytokine syndrome can occur in up to 27% of patients [31]. One patient expired from respiratory distress in a Phase I study in metastatic HER2 cancer patients using third-generation HER2 CAR T cells, and this was ascribed to T cells recognizing low levels of HER2 in lung tissue [35]. Another concern is that long term follow-up may possibly reveal unexpected toxicities from CAR T-cell therapy. Conversely, CD19-directed CAR T cells in B-cell leukemias lead to agammaglobulinemia, a condition easily corrected with gammaglobulin replacement. Targeting cancer germline antigens (e.g., NY-ESO-1, MAGE) is another viable option as these antigens are expressed by healthy cells only during fetal development and not later in life [36]. Methods to minimize or eliminate the negative side effects of CAR T cells include decreasing T-cell doses, the use of suicide gene systems (e.g., HSV-TK, drug inducible caspase-9), and incorporation of defined surface antigens (e.g., CD20) that could be later targeted with mAbs [37].

4. Cell-Based Immunotherapy and Tumor Vaccines

Several T-cell-based immunotherapy strategies are under investigation, such as autologous/allogeneic transplantation of tumor specific CTLs, oncolytic virotherapy, allogeneic NK cell infusions, and tumor vaccines [33, 38]. Adoptive immunotherapy with autologous and/or allogeneic cancer antigen specific CTL has been investigated in both solid and hematologic cancers [39]. Oncolytic virotherapy uses attenuated viruses targeted to specifically infect host cancer cells leading to direct antitumor effects and immunologic cell death from tumor antigen presentation [40]. Killer immunoglobulin- (KIR-) mismatched NK cell infusions are currently under investigation for pediatric leukemias, neuroblastoma, and sarcomas [41, 42]. Several tumor vaccine approaches have also been studied in pediatric cancer, using peptide alone or DC pulsed with tumor peptides or lysates.

EBV-associated posttransplant lymphoproliferative disease (PTLPD)/lymphoma was treated by infusing donor-derived EBV-specific CTL generated using EBV-transformed lymphoblastoid B-cell lines. Infusion of EBV-specific T cells after SCT was found to be highly effective to prevent the development of PTLD and treat preexisting disease [43–45]. EBV-associated tumors express viral antigens and can be targeted using EBV-specific CTL. The association of pediatric nasopharyngeal carcinoma with EBV makes EBV antigens an immunotherapeutic target for cell-based therapy. Several ongoing and recently completed trials utilize either autologous or most closely HLA-matched EBV-specific (LMP-1 and LMP-2) CTL to treat nasopharyngeal carcinoma (NCT00953420, NCT01447056, and NCT00516087).

5. NK Cell-Based Immunotherapy

The antitumor effects of NK cells make them a potential immunotherapy option in the postallogeneic hematopoietic transplant setting and also in the nontransplant setting. Adoptive therapy with NK cells has been carried out to treat AML and several solid tumors, including ovarian cancer, melanoma, breast cancer, renal cell cancer, and advanced lung cancer [46–49]. Due to relatively low numbers in the peripheral blood, immunotherapy with NK cells requires ex vivo expansion to achieve clinically relevant numbers, and, during the expansion process, these cells display increased expression of activation markers, chemokine receptors, and adhesion molecules [50–52]. Further, these studies using ex vivo activated and expanded NK cells have demonstrated extensive cytotoxicity against various tumor cells without affecting the healthy cells. Two pediatric trials (NCT01875601 and NCT01944982) employing ex vivo activated and expanded allogeneic NK cells are ongoing and one trial (NCT00640796) has been recently completed (in 2014). A pilot study on 10 children with AML employed the use of nonactivated, KIR/KIR ligand mismatched haploidentical donor NK cells along with exogenous IL-2 with all 10 subjects remaining in remission 2 years after infusion [53]. A similar study using NK cells in adults reported complete remission in 75% of subjects with KIR/KIR ligand mismatches [48, 54]. There are more than 20 open or recently completed clinical trials employing NK cell-based immunotherapy for pediatric cancers as reported recently in a comprehensive review by McDowell and coworkers [54]. These studies either employ NK cells as monotherapy or in combination with chemotherapy and/or a mAb, such as anti-GD2 antibody. A recent study by Rubnitz and coworkers reported that 76% of children with relapsing or refractory leukemia treated with chemotherapy followed by the infusion of haploidentical NK cells proceeded to hematopoietic cell transplantation and 31% were alive when compared to a parallel study conducted by the same group with only 13% of patients alive with the same chemotherapy, but without NK cells [55, 56].

6. Tumor Vaccines

A major challenge of cancer vaccines is the fact that standard chemotherapy agents can be highly immunosuppressive, limiting the ability of patients to respond to the vaccine [57]. Immunologic adjuvants such as toll-like receptor (TLR) agonists have been used clinically to facilitate both antigen presenting cell and responder cell functions [58, 59]. Another problem is identifying appropriate tumor antigens to target in a vaccine, based on expression patters in individual malignancies. While optimal antigens have not been defined

for many pediatric cancers, several MHC-restricted cancer antigens have been identified on pediatric tumors, of which cancer germline antigens (CGAs) are the most well studied [31]. The earliest evidence for the safety and potential efficacy of cancer vaccines was in malignant melanoma after targeting CGA [60]. Downregulation of MHC Class I and tumor specific antigens is a common mechanism of tumor immune escape, and some highly immunogenic CGAs can be epigenetically upregulated with exposure to demethylating agents (e.g., decitabine, azacytidine). Our group reported the epigenetic upregulation of MAGE-A1, MAGE-A3, and NY-ESO-1 antigens on neuroblastoma and sarcoma cells after exposure to decitabine, thereby enhancing the recognition of tumor cells by antigen-specific CTLs [61, 62].

There are several potential options for cancer vaccines including DC pulsed with tumor lysate, whole tumor proteins, HLA-restricted peptide antigens, and overlapping whole tumor antigen peptide mixes, with or without adjuvants. In one study of children with high-grade glioma receiving vaccines with DC pulsed with tumor lysates, sustained remissions were demonstrated in children with minimal residual disease at the time of vaccination [63]. In another Phase I trial, a 20% response rate was noted with the use of tumor lysate pulsed DC for children with relapsing solid tumors [64]. In a study by Bowman and coworkers, adenovector-mediated transfer of the IL-2 gene into autologous neuroblasts in patients with relapsing neuroblastoma led to a clinically effective antitumor immune response mediated by both helper and cytotoxic T lymphocytes in some patients [65]. The same group later showed that an allogeneic tumor vaccine combining transgenic human lymphotactin with IL-2 in patients with advanced and refractory neuroblastoma led to 2-fold expansion of $CD4^+$ T cells and 3.5-fold expansion of NK cells, inducing a more potent immunologic and clinical response. Twenty-eight percent of the patients had significant increase in NK cytolytic activity and 71% of the patients made IgG antibodies [66]. Currently, there are over 10 ongoing or recently completed vaccine trials for various pediatric solid tumors including pontine and high-grade glioma, medulloblastoma, neuroectodermal tumors, neuroblastoma, and different types of sarcoma [67].

At the author's institution, DC vaccine trials are open incorporating decitabine followed by DC/MAGE-A1, MAGE-A3, and NY-ESO-1 vaccine in the treatment of relapsing/refractory neuroblastoma, sarcomas, and brain tumors. We have previously published our Phase I DC vaccine experience with neuroblastoma and sarcomas wherein one of ten patients achieved a complete response. Two patients were disease-free at start of DC vaccine therapy of which one remains disease-free 2 years off from therapy [68].

7. Summary

There have been several new developments in immunotherapy over the past decade which have dramatically altered the clinical course of children with relapsing or otherwise high risk malignancies. The combination of ch14.18 antibody, aldesleukin, and GM-CSF is a prime example of how mAbs can have a significant impact on patient survival and the notion that immune strategies can safely be incorporated into our standard chemoradiation approach. CAR T-cell therapy shows promise but appropriate target antigens for other tumors besides relapsing pediatric ALL still need to be identified. Tumor vaccines have shown a modest response in some pediatric solid tumors, with better results noted in the setting of minimal residual disease burden and with the use of adjuvants.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Pros and Cons of Antigen-Presenting Cell Targeted Tumor Vaccines

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In therapeutic antitumor vaccination, dendritic cells play the leading role since they decide if, how, when, and where a potent antitumor immune response will take place. Since the disentanglement of the complexity and merit of different antigen-presenting cell subtypes, antitumor immunotherapeutic research started to investigate the potential benefit of targeting these subtypes *in situ*. This review will discuss which antigen-presenting cell subtypes are at play and how they have been targeted and finally question the true meaning of targeting antitumor-based vaccines.

1. Introduction

Active immunotherapy aims to administer the appropriate tumor associated antigens (TAAs) in such a way that antigen-presenting cells (APCs) can process and present them to oncolytic effector cells in order to eradicate primary and metastasized cancer cells. Since the dendritic cell (DC) is the most professional APC, *ex vivo* loaded and stimulated DCs were initially used to achieve this response. However, the *ex vivo* generation and modification of DCs turned out to be a labor-intensive, time- and money-consuming procedure. Furthermore, the variability in DC sources, techniques, and vectors used for TAA transfer led to much diversity in reported TAA expression [1]. Moreover, the *in vitro* generated DCs may not represent the most suited DC subtype for the induction of a $CD4^+$ T helper 1 (T_H1) polarized antitumor immune response. To reduce the DC generation linked variability and exploit the functional characteristics of relevant DC subtypes, naturally circulating DCs have been tested for the induction of long-lasting clinical benefits [2]. Nevertheless, as the isolation of patient-specific DCs remains a labor-intensive and expensive task, direct targeting of TAAs to DCs *in situ* represents a straightforward and therefore preferred strategy. Moreover, direct delivery of cargo to DCs *in situ* could offer additional benefits such as (1) generation of scalable, stable, and standardized vaccines, (2) the ability to tune the direction and strength of the immune response (humoral versus cell-mediated), and (3) improvement of the vaccine's safety profile by reducing the required dose that ends up in nontarget cells and as such diminishing the risk on adverse events. Therefore, numerous groups have evaluated APC targeted vaccination approaches [3–8]. To remain within the scope of this review, we will limit this discussion to APC targeted strategies evaluated in the framework of antitumor immunotherapy.

2. Which Cells Should Be Targeted?

2.1. Dendritic Cells as the Most Professional Antigen-Presenting Cells. The art of antigen processing and presentation to naive T cells *via* major histocompatibility complex (MHC) classes I and II molecules is a privileged feature of three hematopoietic cell types: DCs, macrophages, and B lymphocytes. While the latter two also conduct other functions in innate and humoral immunity, respectively, the former are the most professional and fulltime APCs and are up to 1000-fold more efficient in activating resting T cells [9]. The fact that DCs are specialized APCs is reflected in numerous phenotypic and functional features.

2.1.1. Phenotypically. DCs are characterized by stellate cytoplasmic protrusions, which endow them with an elongated

contact surface for antigen capture and presentation [10]. Their specialized antigen capturing features are further evidenced by the notion of several antigen uptake receptors such as DC inhibitory receptor 2 (DCIR2) and DEC205 [11] next to their unique capability to cross-present exogenous antigens to $CD8^+$ T cells upon uptake of draining antigens and antigen handover from migratory DCs [12] or by acquiring peptide-MHC complexes also known as "cross-dressing" [13]. As opposed to macrophages, they are further able to regulate their processing capacity and by degrading their engulfed cargo more slowly, they can control lysosomal degradation in order to preserve peptides for T-cell recognition [14]. Next to the presence of MHC/peptide complexes, DCs also express several costimulatory molecules in order to properly guide the naive T cells [15].

2.1.2. Functionally. Next to these structural features, DCs have a remarkable functional plasticity. To accomplish this, they are strategically positioned at body barriers and organ entry ports [16]. On the one hand, they are able to induce immune responses against invading pathogens (nonself). On the other hand, DCs can induce tolerance in order to avoid unwanted immune reactions against autoantigens (self) [17]. In general, immature DCs efficiently take up pathogens, apoptotic cells, and particulate antigens from the environment by receptor-mediated phagocytosis, macropinocytosis, or caveolae and clathrin-mediated endocytosis. Furthermore, they remain tissue-resident, have a high turnover rate of MHC-II/peptide complexes, and lack T-cell stimulatory molecules and hence induce T-cell energy instead of T-cell activation upon DC-T cell interaction [18]. In contrast, activated DCs are considered to be immunogenic. Upon maturation, they lose their endo- and phagocytic receptors and slow down their antigen capture and processing rate, while they upregulate both "signal two" molecules like costimulatory molecules (e.g., CD80 and CD86) and "signal three" molecules (e.g., IL-12) to stimulate and polarize naive T cells, respectively. Furthermore, they acquire a higher cellular motility by upregulating the C-C chemokine receptor type 7 that enables DCs to migrate from the periphery to the T-cell areas of draining lymphoid tissues. However, the view that immature DCs induce tolerance and mature DCs induce immunity is simplified. It has been demonstrated that mature DCs can contribute to T-cell tolerance as well [19], suggesting that the maturation trigger dictates the immune functions of the DCs.

2.1.3. Sensitivity. The third reason why DCs are such sophisticated APCs is reflected by the complexity of maturation signals they can detect and respond to [14]. The most important pathways known today are (1) the encounter of microbial agents that trigger surface or intracellular Toll like receptors (TLRs), C type lectin receptors (CLRs), retinoic acid-inducible gene 1 (RIG-I) or nucleotide-binding oligomerization domain (NOD) like receptors [20, 21], (2) the direct interaction with cells such as B cells, T cells, natural killer (NK) cells, natural killer T (NKT), and $\gamma\delta$ T cells, (3) stimulation by cellular products like CD40 ligand (CD40L), IL-1β, TNFα, and IL-6, and (4) the products of dying cells named damage-associated molecular patterns (DAMPs) like heat shock proteins, high mobility-group box 1 proteins, and uric acid [17]. As distinct antigens are able to trigger DC maturation *via* one or more of these pathways, this combination serves as a fingerprint that triggers a specific set of receptors [22, 23]. Subsequently, complex signaling networks are activated which cooperate, integrate and finally converge in the upregulation of distinct transcription factors [24].

A final hallmark of their professionalism is represented by their differential anatomical locations, expression of different markers, distinct antigen processing capacities, and variable responses to maturation stimuli or, in other words, their subdivision in specialized subtypes, as discussed in the next section.

2.2. Dendritic Cells as a Heterogeneous Population of Subsets. Given the plethora of antigens, their varied routes of entry into the body, and their diverse characteristics, it is not surprising that a network of professional APCs dedicated to control T-cell immunity diversified to cope with all intruders at all phases of the immune response. About 15 years ago, researchers started to investigate the complexity and merit of the different DC subtypes. However, unraveling this complexity has been complicated in part due to the rarity of DCs in tissues (≈1% of cells), their short life span, and their lack of cross-species unifying surface markers. Therefore, the field of DC subtyping came with a lot of observations, hypotheses, and contradictions. However, with the latest ontogenic, phenotypic, and genetic data, it is currently postulated that there are two main "true" DC subtypes in both mice and men: plasmacytoid DCs (pDCs) and conventional DCs (cDCs), which are further subdivided into cDC1 and cDC2. Below we will elaborate on these subtypes as well as on two cell types that were long considered to be distinct DC subtypes, namely, Langerhans cells (LCs) and monocyte derived DCs (moDCs).

2.2.1. Ontogenic Level. "True" DCs are defined by their fms-like tyrosine kinase 3 ligand- (Flt3-L-) dependent development from hematopoietic stem cells into blood residing preDCs and pDCs [25]. Next, the development of cDC1 is orchestrated by IFN regulatory factor-8 (IRF8), basic leucine zipper ATF-like 3 transcription factor (BATF3), nuclear factor regulated by interleukin-3 (NFIL3), and inhibitor of DNA binding 2 (Id2) [26]. When Id2 is suppressed by E2-2, pDCs are generated [27]. To differentiate into cDC2, transcription factors v-rel avian reticuloendotheliosis viral oncogene homolog B (RelB), neurogenic locus notch homolog protein 2 (NOTCH2), recombination signal binding protein for immunoglobulin kappa J region (RBP-J), IRF2, and IRF4 are employed [28]. Although presumed for a very long time, both skin residing LCs and moDCs are not considered "true" DCs since their development is Flt3-L-independent [29]. While circulating monocytes are rapidly mobilized to differentiate into moDCs under inflammatory conditions [30], LCs seem to originate from fetal liver monocytes [31] that require colony stimulating factor 1 receptor (CSF1R) engagement *via*

IL-34, which suggests that LCs are more closely related to macrophages [26].

2.2.2. Phenotypic Level. Initially both murine and human cDCs were defined as $CD11c^+$ $MHC\text{-}II^+$ cells located in lymphoid as well as nonlymphoid tissues. Furthermore, cDCs found in lymphoid tissue like bone marrow, spleen, and lymph nodes are called resident DCs and were subdivided into $CD8\alpha^+$ or $CD4^+$ cDCs in mice versus CD1c (BDCA-1)$^+$ or CD141 (BDCA-3)$^+$ cDCs in human. In the nonlymphoid tissues like skin, lungs, and gut, DCs are called migratory since they tend to migrate from peripheral tissues to lymphoid tissue through the lymphatics. In mice, these cDCs are defined to express CD103 or CD11b while, in humans, the same surface markers as in the lymphoid tissues were observed. So while in mice cDCs express different markers in different anatomical locations, human $CD141^+$ DCs and $CD1c^+$ DCs are abundantly present in both lymphoid and some nonlymphoid tissues such as liver, lung, and skin. Next to the cDCs, pDCs are also broadly distributed throughout the body [32]. While mouse pDCs are $Lin^-MHC\text{-}II^+$ and specifically express CD11c, B220 (CD45R), CD317 (BST2), and SiglecH, human pDCs express IL-3Rα (CD123), CD303 (BDCA-2), and CD304 (BDCA-4). The murine moDCs express CD11b, CD11c, MHC-II, CD64, and FcγRε alongside varying levels of Ly6C. The human counterparts all express high levels of MHC-II, CD11c, CD11b, CD24, CD1a, and CD206 but lose expression of both macrophage colony stimulating factor (M-CSF) receptor and Ly6C [33]. Of note, an extra subset of human dermal DCs is represented by the $CD14^+$ cells which are characterized by their expression of DC-specific ICAM3-grabbing nonintegrin (DC-SIGN), C-type lectin domain family 1 member (CLEC) 6, lectin-like oxidized LDL receptor-1 (LOX-1), and dectin-1 [34]. Finally, murine LCs characteristically express langerin (CD207), a C type lectin that is localized in LC-specific organelles called Birbeck granules. Human LCs are identified as $langerin^+$, $DEC205^+$, $CD1a^{hi}$, and $CD11c^{lo}$ (Table 1).

2.2.3. Genetic Level. It became clear that murine lymphoid tissue $CD8\alpha^+$ and nonlymphoid tissue $CD103^+$ cDC subsets as well as the lymphoid tissue $CD4^+$ and nonlymphoid tissue $CD11b^+$ DC subsets combined constitute two cross-species DC lineages, respectively. Therefore, it was recently proposed to subdivide cDCs into only two main subtypes: one classical type 1 DC (cDC1) for murine $CD8\alpha^+/CD103^+$ and human $CD141^+$ cDCs [35] and cDC2 for murine $CD4^+/CD11b^+$ and human $CD1c^+$ cDCs (Table 1). Within the cDC1 group, chemokine receptor-1 (XCR1) is emerging as an important cross-species marker, which supports the view that the traditional DC subset markers CD8α and CD141 are inferior identifiers of the cDC1s and are being superseded by XCR1, CLEC9A (DNGR-1), and cell adhesion molecule 1 (CADM1). Within the cDC2 group, the most conserved markers are $MHC\text{-}II^{hi}$ and signal-regulatory protein α ($SIRP\alpha^+$). The only known pDC-specific conserved markers are IRF7/8 next to TLR7/9 while the conserved markers of LCs are MHC-II, E-cadherin, epithelial cell adhesion molecule (EpCAM), CD11c, and langerin (CD207). Finally, while the human and murine moDCs share the conserved markers MHC-II, CD11b, and CD11c, the human moDCs are further characterized by CD16 and the murine version by Ly6C and DC-SIGN.

2.2.4. Functional Level. Both murine and human cDC1 selectively express genes involved in the balance between tolerance and cross-presentation [36, 37]. They express high levels of MHC-I processing-associated proteins. In addition they possess the dual capacity to produce large amounts of type I IFN and IL-12, making cDC1 ideal stimulators of $CD8^+$ cytotoxic T cells (CTLs) [14, 38]. A recent study underlined that only the intratumoral cDC1s were able to facilitate adoptive CTL control of tumor outgrowth [39].

In contrast mouse and human cDC2 efficiently present antigens to $CD4^+$ T cells, favoring their polarization into T_H2 and T_H17 cells. They also appear to display a capacity to cross-present antigens and secrete high levels of IL-12, suggesting a potential key role in promoting IFNγ release by NK cells and therefore also T_H1 polarization [40]. This redundancy for cDC1 and cDC2 may be a way to allow "mass cross-presentation" as the human $CD141^+$ cDC1 represent only a small fraction (≈2%) of all DCs, at least in blood [41].

The pDCs are best known for their ability to produce high amounts of type I IFN (IFNα and IFNβ) in response to viral stimuli and as such control the progress of viral infections at various levels [42]. In their resting state, however, pDCs play an important role in the induction of tolerance owing to a low expression of MHC and costimulatory molecules compared to their cDC counterparts. However, in humans this view has been challenged by recent findings that metastatic melanoma patients receiving intranodal injections of activated and peptide loaded pDCs were very effective at inducing potent antitumor immunity [43].

MoDCs are a special type of subset since they are created according to the type of inflammation. In general, moDCs capture antigen and migrate to the draining lymphoid tissues to predominantly drive T_H1 or T_H17 immunity by producing IL-12 or IL-23, respectively. After infection they can also produce TNFα and inducible nitric oxide synthase (iNOS). Furthermore, they seem to be evolved as a crucial reservoir of APCs with a potent emergency backup role in cases of acute inflammation [30].

Finally the murine epidermal LCs are an atypical APC subset that seems specialized in the uptake and processing of antigens in the periphery for peripheral tolerance induction, especially during steady state conditions. In addition, they can produce IL-23, IL-6, and IL-1β during inflammation. On the contrary, human LCs have been described to induce robust proliferation of naive allogeneic $CD8^+$ T cells far more efficient than the $CD14^+$ DCs through the secretion of IL-15 which promotes the differentiation of granzyme B^+/$perforin^+$ CTLs. Moreover, they appear to be efficient at cross-presenting peptides [44]. In general, the role of LCs seems to be dictated by environmental cues, rather than a preimprinted behavior.

In summary every DC subset has its own functional specialties, which opened up exciting possibilities for targeted manipulation to tune the immune response by harnessing

Table 1: Concise overview of the ontogenic, phenotypic, and functional features of the five main DC subtypes: cDC1, cDC2, pDC, LC, and moDC.

	cDC1	cDC2	pDC	LC	moDC
Ontogeny	HSC + Flt3-L, BATF3, NFIL3, and Id2	HSC + Flt3-L, RelB, NOTCH2, RBP-J, IRF2, and IRF4	HSC + Flt3-L and E2-2	Blood residing monocytes + inflammation	Fetal liver monocytes + CSF1R
Mouse Other markers	CD8α^+/CD103$^+$ cDC DEC205$^+$	CD4$^+$ CD11b$^+$ cDC	SiglecH$^+$ BST2$^+$ pDC B220$^+$	Langerin$^+$ LC	CD11b$^+$ moDCs CD64, FcγRε, and Ly6c
Human Other markers	CD141$^+$cDC CD162^{hi} DEC205^{hi}	CD1c$^+$cDC CD11b$^{lo/+}$	CD123$^+$ pDC BDCA-2$^+$, BDCA-4$^+$	Langerin$^+$ LC DEC205, CD1a^{hi}	CD11b$^+$ CD1a$^+$ moDCs CD24$^+$, CD206$^+$, CD16$^+$, and DC-SIGN
Conserved (besides CD11c and MHC class II)	TLR3$^+$ CADM1$^+$ XCR1$^+$ CLEC9A$^+$	MHCII^{hi} SIRPα^+	TLR7^{hi} TLR9^{hi}	E-cadherin$^+$, EpCAM$^+$, and langerin$^+$	CD11b$^+$
Functions	T_H1 Cross-presentation	T_H2 and T_H17 Cross-presentation	IFN-α/β and IFNλ Humoral	Adaptable MOUSE: Treg or T_H17 HUMAN: IL-15 promoting CTLs + Cross-presentation	Highly adaptable (IL-12, IL-23, TNFα, and iNOS)

subset specific attributes. Subsequently antitumor vaccination became not only a question of proper DC activation but also of selecting the most appropriate DC subtype [34, 44].

3. How Can Antigen-Presenting Cell Subsets Be Targeted?

In general every active antitumor vaccine needs to comprise both a TAA and an appropriate stimulus to avoid the induction of TAA specific tolerance. In terms of vaccination modalities, we can roughly subdivide them in four groups: naked protein based, naked nucleic acid based, viral vector based, and nanoparticle based vaccines [40, 45–48]. In general, both naked protein and nucleic acid based vaccines are relatively easy to generate. However, they always need to be codelivered with an adjuvant to achieve robust antitumor immunity. On the contrary, viral vectors and nanoparticles are intrinsically immunogenic as they have a pathogen-like size and appearance. Moreover, when *in vivo* vaccination of mice with a viral vector was compared to peptide, DNA, or DC vaccination, stronger tumor specific immune responses were elicited with viral vectors [49–51]. As antitumor vaccines have been developed in numerous shapes and sizes, their extent of targeting possibilities is very diversified as well. In this section we will discuss the three main targeting approaches while a detailed overview of the performed preclinical and clinical *in vivo* APC targeting experiments in the framework of antitumor immunotherapy is summarized in Table 2.

3.1. Administration Based Targeting. After antigen delivery the so-called "depot-effect" tends to retain most of the antigen at the injection site. To increase vaccine uptake by APCs, the most straightforward way is represented by vaccine delivery into an APC rich site such as the tumor draining lymph node or spleen. For example, when we delivered TAA encoding lentiviral vectors (LVs) or mRNA intranodally in mice, a stronger therapeutic CD8$^+$ T-cell response was induced than after subcutaneous delivery [45, 52]. Alternatively vaccines can be developed in such a way that they become prone to accumulation in lymphoid organs [53–55]. The latter is exemplified by two different studies in tumor bearing mice with nanoparticles (NPs) coupled to adjuvant alone or also a TAA. The NPs accumulated in the tumor draining lymph nodes when intradermally administered in the limb ipsilateral to the tumor or in the nontumor draining lymph node when administered in the contralateral limb. Interestingly, only when these NPs were targeted to the tumor draining lymph node, the CD4$^+$ T-cell distribution within the tumor repolarized towards a T_H1 phenotype and an increased frequency of therapeutic antigen-specific CD8$^+$ T cells within the tumor was observed. Together, these data implicate that the tumor draining lymph node is an appealing vaccine target for solid tumors and can be targeted with NPs [56–58]. Of note also skin DC networks have been targeted *via* the use of polymeric dissolving microneedle arrays with nanoencapsulated antigen [59, 60].

Recently, intratumoral administration of antitumor vaccines has emerged [61]: on the one hand because numerous vaccination studies showed the induction of potent TAA-specific T-cell responses without clear therapeutic benefit [62] and on the other hand because the tumor microenvironment turns out to be a very manipulative system that is able to protect tumor cells from a cytotoxic attack and moreover help in tumor progression. Noteworthy in this process are the regulatory myeloid cells, represented by myeloid derived suppressor cells, type 2 or N2 tumor associated neutrophils, a subset of mast cells, M2 macrophages, and regulatory tumor associated DCs [63]. Although the latter two could

Table 2: Summary of *in vivo* APC targeting studies in the framework of antitumor vaccination.

Targeting moiety	Injection	Content	Effect	References
		CLR		
DEC205 α-GalCer NP	fp	OVA	↑iNKT, ↓growth in B16F10, and EG7-OVA (P + T)	[147]
Selected nucleic acid aptamer	i.v.	OVA	↑CD8, ↓growth OVA-B16 tumor (T if OT-I transfer)	[95]
Anti-CD11c and DEC205 scFv coupled to NP	i.v.	OVA + ADJ	↑CD8, ↓growth OVA-B16 tumor (P)	[83]
mAb fused protein	s.c.	OVA + ADJ	↑CD8, ↓growth OVA-B16 (P + T)	[131]
mAb fused protein	i.p.	HER2 + ADJ	↑CD8, ↑CD4, ↑humoral, and ↓growth neu-expressing mammary tumor (P)	[148, 149]
mAb fused protein	i.p.	Mesothelin + ADJ	↑cross-presentation, ↑CD4, ↑humoral, and ↓growth neu-expressing mammary tumor (P)	[150]
scFV modified adenoviral vector	fp	OVA	↑T cell, ↑humoral (at low doses), ↓growth OVA-B16 (P) BUT better for untargeted vectors	[127]
mAb fused protein	fp	OVA + ADJ	↑CD8, ↓growth B16 pseudo-metastasis model (P + T)	[120]
Bacteriophage displaying scFV	fp	OVA	↓growth B16F10 (Pro + Ther)	[151]
mAb fused protein	s.c.	Trp2 and gp100 + ADJ	↑CD8, ↑CD4, and ↓growth B16 melanoma (P + T)	[152]
scFV fused to DNA vaccine	**i.m.**	**Her2/neu + CPM**	**↑CD8, ↑humoral, long lasting memory ↓growth HER2/neu$^+$ D2F2/E2 breast tumor + spontaneous mammary carcinomas (P + T)**	[153]
Phase I clinical trial with CDX-1401 = human mAb fused protein	**i.d.**	**NY-ESO-1 + ADJ**	**Patients with advanced malignancies: ↑cellular, ↑humoral (T)**	[154]
DEC206 Mannosylated NP	s.c.	OVA + ADJ	↑T_H1 cell, ↑humoral, and ↓growth B16F10 (P + T)	[155]
mAb fused to protein	s.c.	OVA + ADJ	↑T cell, ↑humoral, and ↓growth B11-OVA (P)	[156]
Mannan coupled protein	i.p.	MUC-1	↑CD8, ↓growth P815 mastocytoma (T)	[157]
Mannose coupled dendrimer	i.d.	OVA	↑CD8, ↑CD4, ↑humoral, ↓growth B16-OVA (P)	[158]
Mannosylated NP	s.c.	ErbB2/HER3 + ADJ	↓growth huErbB2$^+$ renal carcinoma cells (T)	[125]
Mannan coated liposome-protamine-DNA	U	HPV16 E7	↓growth E7$^+$ TC-1 (P + T)	[159]
Mannosylated and/or histidylated NP loaded with mRNA	i.v.	MART-1	↑CD8, ↓growth B16F10 (P)	[160, 161]
Mannan or pullulan NP complexed with protein	U	HER2	↑CD8, ↓growth HER2$^+$ tumors (P + T)	[162]
D-mannose conjugated lipid-core peptide system	s.c.	HPV16 E7	↓growth TC-1 HPV-16 tumor (P)	[163]
Clinical trial with mannan coupled protein	**s.c.**	**MUC-1**	**↑humoral, less ↑CD8, protection against recurrence in breast cancer patients**	[106]

Table 2: Continued.

Targeting moiety	Injection	Content	Effect	References
Two phase I studies with CDX-1307 = hCG-β fused to mAb	**i.d. or i.v.**	**hCG-β + ADJ**	**↑humoral and T cell with clinical benefit in patients with advanced epithelial malignancies**	[164]
DC-SIGN LV pseudotyped with point-mutated Sindbis virus glycoprotein	i.d.	OVA or PSCA	↑CD8, ↑CD4, and ↓growth transgenic adenocarcinoma, E.G7-OVA and PSCA-expressing B16-F10 (P + T)	[5, 103, 165]
mAb coupled protein	U	KLH	↓growth human Burkitt's lymphoma cell line in humanized mice (P)	[166]
IDLV pseudotyped with engineered Sindbis virus glycoprotein + **currently tested in Phase I clinical trial ID-VP02**	s.c.	NY-ESO + Vpx	↑CD8, ↓growth CT26 colon carcinoma cells (P + T)	[167, 168]
LOX-1 HSP70 fused to protein	s.c.	OVA	↑CD8 and cross-priming, ↓growth E.G7 cells (P)	[169]
CLEC9A mAb coupled to peptide	s.c.	MUC-1 + ADJ	↑T_H1, ↓growth MUC-1-A2K/b$^+$ MC38 (P + T)	[170]
mAb coupled to peptide	s.c.	OVA + ADJ	↑CD8, ↓growth B16 lung pseudo metastases (P + T)	[138]
DCIR2 Anti-DCIR2 or anti-DEC205 mAb coupled protein	i.p.	OVA + ADJ	↑CD8, ↑CD4 (mixed T_H1/T_H2), ↑humoral, and ↓growth B16F10-OVA (P + T)	[171]
		Integrin		
CD11c Targeted lipopeptide	i.d.	OVA, WT1, tumor lysate + ADJ	↓growth for OVA: E.G7-OVA, for mWT1: mWT1–1498 cells and for tumor lysate: MHC-I$^-$ B16D8 melanoma (T)	[172]
Tumor-derived plasma membrane vesicles engrafted with two CD11c binding peptides	i.v.	OVA	↑CD8, ↑humoral, and ↓growth of metastatic B16-OVA (T)	[173]
CD11b Adenylate cyclase-(CyaA-) based vector	i.p. vs. i.v. or i.d.	OVA vs. HPV E7	↓growth OVA-B16 or E.G7-OVA versus TC-1 (P + T)	[113, 174]
Phase II study with ProCervix = CyaA-based vector	**s.c.**	**HPV16 and 18 E7 + ADJ**	**Clinical phase I trial indicated good safety and local tolerance at the highest dose, ↑T + ↑viral clearance + controlled HPV recurrence**	NCT01957878
		Fcγ receptor		
IgG1-Fc tumor cells	s.c.	TAAs	↓growth E.G7 (P + T)	[175]
HER2-Fc cDNA	i.m. + EP	HER2	Mu: ↑T, ↓growth HER2$^+$ D2F2/E2 cells (P) **Hu: *in vitro* cross-processing and ↑CD8$^+$ T cells from breast cancer patients**	[176]
		MHC-II molecule		
DNA loaded dendrimer with targeting peptide	s.c.	Trp2 or gp70 vs. OVA	↑CD8, ↑humoral, ↓growth, strong for B16OVA, and weak for gp70 BUT better with EP (P) ↔ B16 with Trp2 (T)	[177]
LV pseudotyped with scFv coupled to H protein of measles virus envelope	i.v.	OVA or male HY gene	↑CD4, ↑cytotoxic, and memory CD8 BUT not to the same extent as broad tropism LVs	[112, 178]
LV pseudotyped with scFV coupled to murine leukemia virus envelope	s.c.	OVA	↑CD8 mediated IFNγ secretion	[179]

Table 2: Continued.

Targeting moiety	Injection	Content	Effect	References
DNA encoding anti-MHC II and anti-CD40 scFv or chemokines (MIP-1α, RANTES) with scFV of idiotype	i.m. or i.d. + EP	Idiotypes	↑CD8, ↑humoral, and ↓growth Id^+ tumors (P)	[180, 181]
		Non-CLR surface marker		
BST2 Protein fused anti-BST2 Ab	i.p.	OVA or pHEL + ADJ	↑CD4, ↑CD8, ↑humoral + ↓growth B16-OVA (P)	[182]
		Undefined		
NP with cholesteryl pullulan towards medullary macrophages	s.c.	MAGE-A4 or mERK2 + ADJ	↑cross-presentation, ↓growth MAGE-$A4^+$ $CMS5^+$ CT26, and $mERK2^+$ sarcoma cell line (P + T)	[183]
Listeria monocytogenes expressing TAAs	i.p.	VEGFR2 + ADJ	↑CD8 with epitope spreading, ↓growth breast tumors (P + T)	[114, 184]
Coronavirus vector	i.v.	MelanA or Gp33 + ADJ	↑CD8, expanded epitope repertoire, growth $MelanA^+$ or $gp33^+$ B16F10 (P + T)	[185, 186]
APC specific nanobody displaying LV	i.n.	OVA	↑CD4, ↑CD8, ↓growth (T) BUT not to the same extent as broad tropism LVs	[45]
ISCOM vaccine	s.c.	OVA	↑CD8, ↓growth EG-7-, B16-, or Panc-OVA (P)	[187]
		Costimulatory molecule		
CD40 PLGA-NP coated with mAb	s.c.	OVA and E7 + ADJ	↑CD8, ↑CD4 + ↓growth B16-OVA (P + T)	[188]
CD40 targeted adenoviral vector	i.p.	PSMA + ADJ	↑CD8, ↓growth RM-1-PSMA model (T)	[189]
CD40L extracellular domain to adenoviral vector in mice + **Clinical trial**	i.d.	Mice: Trp2 or gp100 **Human: MART-1**	Mice: ↑CD8, ↓growth B16F10 (T) **↑CD8 in melanoma-draining sentinel lymph nodes**	[190, 191]
B7 Syngeneic epithelial cells continuously secreting CTLA-4-ErbB2 fusion vaccine	s.c.	HuErbB2 + IL-15	↑CD8, ↑humoral, ↓growth $ErbB2^+$ renal cell carcinoma (T)	[192]
Treml4, Ig superfamily member mAb against Treml4	i.p.	OVA or HER2 + ADJ	↑CD8, ↑CD4, ↓growth neu^+ mammary tumor cell line NT2.5 (P)	[193]
TLRs TLR9 targeting protein (via DNA sequence)	i.d.	OVA + CpG	T_H - independent ↑CD8 + ↓growth E.G7-OVA (P + T)	[194]
TLR2 targeting lipid moiety + epitopes	s.c.	OVA	↑CD8, ↑humoral + ↓growth B16-OVA, and Lewis lung-OVA (P + T)	[195]
TLR5 targeted peptides (via flagellin) engrafted onto liposomes	i.v.	OVA	↑maturation of DCs, ↑CD8, ↑humoral, ↓growth B16, and P815 (P + T)	[173, 196]
TLR4 targeting protein (via fibronectin)	i.t. or i.v.	HPV E7 w or w/o ADJ or CPM	↑CD8 with cure of established TC-1 tumors i.t.: in the absence of additional ADJ i.v.: when + ADJ or CPM + ADJ	[197]
TLR4 targeting protein (via fibronectin) + anti-CD40, TLR3 and TLR7 ligands	s.c.	OVA + ADJ	↑CD8, ↓growth B16-OVA or B16.F10 (T)	[198]

Table 2: Continued.

Targeting moiety	Injection	Content	Effect	References
Chemokine related Fusion of chemokine MCP3 or IP10 to lymphoma-derived scFv as protein or DNA plasmid	s.c. or i.d.	scFV	↑humoral, ↓growth 38C-13 and A20 (P)	[199]
OVA with mAb or chemokine ligand XCL1 against XCR1	i.v.	OVA + ADJ	↑CD8, ↓growth E.G7 (P)	[200]

P: prophylactic, T: therapeutic, fp: footpad, i.v.: intravenous, s.c.: subcutaneous, i.p.: intraperitoneal, i.m.: intramuscular, EP: electroporated, i.d.: intradermal, i.n.: intranodal, i.t.: intratumoral, U: unknown, ADJ: adjuvant, CPM: cyclophosphamide iNKT: induced natural killer T cell, and in bold: all studies with human APCs.

be potent antigen presenters of the TAAs they capture in their surroundings, the tolerogenic microenvironment squeezes them into a suppressive state. Interestingly, since they retain a highly plastic phenotype, it seems possible to reprogram them towards potent antitumor immunity stimulating APCs. Therefore, our understanding is shifting emphasis from targeting APCs within the draining lymphoid organs by intranodal injection or lymph node targeted NPs to reprogramming APCs within the tumor microenvironment by intratumoral injection [64].

While administration based targeting is a very straightforward way to increase the chance that APCs are stimulated, it does not allow APC subtype specific targeting. Therefore, other targeting approaches have been developed as discussed in the following paragraphs.

3.2. Expression Based Targeting. When a nucleic acid based vaccine is administered, the expression of the encoded TAAs is most often driven by a strong constitutive promoter with or without enhancer sequences. These include the cytomegalovirus, spleen focus forming virus, human polypeptide chain elongation factor-1α, phosphoglycerate kinase, and ubiquitin C promoters [65–67]. Although these promoters induce strong and ubiquitous expression of the transgene, they are (1) more prone to promoter inactivation than cell specific promoters, (2) more potent in activating the host-cell defense machinery, and (3) increasing the potential risk of insertional mutagenesis caused by their enhancer sequences [68, 69]. These downsides resulted in the development of various strategies to allow APC-specific transgene expression by incorporating cell type specific regulatory elements and/or promoter(s) in the expression cassette [70, 71]. Examples are the CD11c, DC-SIGN, DC-STAMP, langerin, HLA-DR, MHC-II, and dectin-2 promoter [72–75]. However, DC specific transgene expression does not guarantee a strong CTL response, since DC specific promoters have also been applied to induce transgene specific tolerance [76, 77]. Furthermore, it has been described that tissue specific promoters may still be active in many different cell types or states since the promoter is used outside its normal genomic context [78]. Moreover, transcriptional targeting does not reduce the possible risk for insertional mutagenesis nor the possibility of cargo transfer to germ line cells [79]. Due to these conflicting outcomes, more research needs to be done on the immune stimulatory potential of APC specific promoter driven antitumor vaccines.

3.3. Cell Entry Based Targeting. Cell entry based targeting exploits APC specific surface receptors to target a particular APC subtype and mediate vaccine internalization [80]. Robustly, five main APC specific receptor families have been evaluated for targeting: the CLR family, integrins, Fcγ receptors, MHC-II molecules, and immune stimulating receptors. Of these, CLRs have been the focus of most APC targeted research in mice, nonhuman primates, and humans (Table 2) [81, 82]. Typically CLRs recognize carbohydrate structures in a calcium-dependent manner and are as such involved in the recognition and internalization of many glycosylated self-antigens and pathogens. Subsequently, CLRs can facilitate antigen uptake, processing, antigen routing, and MHC-I and -II loading. Furthermore, we depict in Table 2 all non-CLR, noncostimulatory molecular targets, as well as costimulatory molecules, used to restrict antigen delivery to APCs on the one hand and license the APC on the other hand. In general, studies regarding the most suited receptor for the induction of potent antitumor immunity remain thus far very contradictory. For example, when mice were immunized with liposomes coupled to single chain Ab fragments (scFv) against CD11c or DEC205, the latter performed twice as good [83]. In contrast, it has also been shown that CD11c targeting was better than targeting CD205, MHC-II, CD11a, CD11b, DCIR2, or CD40 in terms of cellular and humoral immunity [40].

Because receptor ligation influences intracellular vaccine routing, receptor selection has important functional consequences concerning antigen presentation and T-cell stimulation [84]. Consequently, it seems a matter of not only targeting the most suitable DC subtype, but also targeting the most appropriate DC specific receptor to induce a tailor-made response [85]. More recently, three receptors unique for the cDC1 subset have been identified, namely, DNGR1 or CLEC9A, CADM1$^+$, and XCR-1. Interestingly, they are all conserved molecules across different species that are also mechanistically involved in the antigen cross-presentation process [86]. Where CLEC9A is involved in the uptake of antigen derived from apoptotic/necrotic cells, CADM1 binds to CD8$^+$ T cells and mediates DC:CD8$^+$ T-cell adhesion, while XCR1 promotes the functional interaction of cDC1 with NK cells and CD8$^+$ T cells that secrete XCL1 [87, 88]. Since the cross-presenting cDC1 subset is currently seen as one of the most suitable targets for antitumor immunotherapy, targeting vaccines towards one of these receptors holds great promise for antitumor vaccination [39, 88].

Besides the considerable diversity in APC specific receptors, also in the approach to target these receptors there appears to be plenty of choice.

Based on the overview in Table 2, we can conclude that most studies target their vaccine by coupling it to a short peptide, a ligand, a mAb, or carbohydrate. While the latter has been used extensively to target DCs *in situ*, they mainly rely on CLR binding, which results in APC but not DC subtype specific binding. On the contrary, the former three moieties could be generated to bind one particular APC subset specific receptor. Other advantages of short peptides are that they do not severely disrupt the original vaccine formation and that targeted peptides with strong binding affinity and unlimited specificity could be generated *via* high-throughput library approaches [89, 90]. However, they can hinder multimerisation of monomers, create fusion products with lower thermostability, and hinder proper intracellular trafficking of the vaccine [91]. Alternatively, different kinds of ligands such as cytokines and growth factors have been used [92, 93]. In addition to peptides and ligands, also mAbs and their derivatives have been evaluated for APC specific targeting. In general, scFvs offer higher specificity than short peptides but as they are larger in size, the chance that they disrupt the process of conformational changes to mediate membrane fusion increases. Therefore, scFvs are most often linked to a spacer peptide or protease cleavable peptide that permits proper conformation of both the scFv domain and targetable vaccine [94].

Recently described alternatives to the above-mentioned targeting moieties are designed ankyrin repeat proteins (DARPins) and nucleic acid based aptamers as they can be selected to become high-affinity binders to any kind of target molecule [95, 96]. Another interesting alternative lies in the antigen binding part of heavy-chain-only Abs which are found in members of the family of Camelidae [97, 98]. These antigen-binding parts are only composed of one single variable region, termed VHH or nanobody. These nanobodies have unique characteristics and offer many advantages over scFvs such as (1) high solubility, (2) ability to refold after denaturation whilst retaining their binding capacity, (3) cloning and selection of antigen specific nanobodies obviating the need for construction and screening large libraries, (4) nonimmunogenic, and (5) being fused to other proteins [99, 100]. Therefore, it is not surprising that several studies have reported on the generation and subsequent use of nanobodies for APC targeting. Examples hereof are the development of nanobodies targeting CD206 to enabling selective targeting of the MMR^{hi} M2 macrophage subset within solid tumors [101]. Furthermore, we also demonstrated that several nanobodies with yet unidentified target antigens allowed targeting of specific human and murine APC subsets, including DCs and macrophages or selective targeting of cDCs [102].

In the case of viral vectors, several additional strategies have been evaluated to alter the broad infection profile of the viral outer membrane embedded glycoproteins towards an APC specific tropism. A first strategy is represented by rational point and domain mutations of the viral glycoprotein. This is exemplified by the DC-SIGN-specific targeting strategy that is based on the fact that the Sindbis virus envelope glycoprotein consists of a fusogenic E1 protein and a cell binding E2 protein. E2 normally binds to the DC-SIGN receptor, next to the canonical viral receptor heparin sulphate, expressed by many cell types. Since both protein binding sites are physically separated, selective mutation at the E2 monomer is possible, abrogating the heparin sulfate binding part while leaving the DC-SIGN binding part intact. By pseudotyping a LV with this mutated Sindbis virus derived envelope glycoprotein, targeted infection of murine DCs after direct subcutaneous administration was achieved. Moreover, this elicited strong and therapeutic antigen specific immune responses [5, 103–105]. Besides genetic alterations, the viral surface can also be chemically engineered to alter the binding specificity. Advantages are the flexibility, speed, and controllable modification conditions [106–108]. Unfortunately, the effectiveness of the chemically modified particles strongly depended on the reaction conditions of the applied modifications [109, 110]. A final strategy to generate APC targeted LVs is based on the fact that binding and fusion functions of LVs can be separated over two distinct glycoproteins. Recently we exploited this concept to develop DC subtype specific LVs by pseudotyping them with a fusogenic but binding defective glycoprotein on the one hand and an APC specific transmembranary nanobody on the other hand. Briefly we demonstrated cDC or also pDC and macrophage specific transduction of human subsets *ex vivo* and murine subsets *in vivo* [4, 111]. Importantly, similar to the report of Ciré et al. [112], who used a MHC-II targeted approach, we showed that intranodal administration of DC targeted LVs enhanced $CD4^+$ T-cell proliferation, without functional nor therapeutic benefit compared to untargeted LVs [45]. Of note, besides viral vectors also bacterial derived vectors or enzymes have been successfully used to target TAAs to DCs with subsequent maturation and induction of strong antitumor immunity [113–115].

In summary an enormous amount of studies have been performed to evidence the added value of DC targeting for antitumor vaccination. However, most receptors (DEC205, DEC206, DC-SIGN, DCIR2, LOX-1, CD11c, CD11b, Fcγ receptors, and MHC-II molecules) are not truly specific for one particular APC subset. Subsequently they have been described to internalize antigen by different DC subsets, different APC subsets, and even other non-APCs such as endothelial cells and thymic epithelial cells [8, 116], which hampers the evaluation of the DC subtype specific impact on the induced immune response. In addition, several conflicting reports were made in mouse versus human related studies using homologous targeting moieties [117]. For example, when MUC1 was targeted to CD206, a robust CTL response was induced in mice while a robust humoral but only moderate T-cell response was observed in adenocarcinoma patients [106]. In addition, it is difficult to compare the different APC targeting studies since they were performed with very different vaccine moieties with different sizes and surface charge, different doses, diversified formulations, and targeting approaches which can result in completely different pharmacokinetic and immunological outcomes [47]. Therefore, there is an urgent need to define the true meaning of DC

subset specific targeting to serve our understanding of potent active vaccines for antitumor immunity [118].

4. Is Targeting a Step Forward in Vaccine Development?

Although numerous studies evaluated and confirmed the efficacy of DC subtype specific targeting for immunotherapeutic purposes, other studies question its improvement compared to untargeted delivery [40, 45, 119]. Therefore, we want to elaborate in this section on the true meaning of DC (subtype) specific targeting for active antitumor vaccination.

First of all, it has been questioned if it is really possible to target APCs "actively" as they are already specialized in the uptake of whatever antigen they encounter. When Kreutz et al. injected an anti-DEC205 Ab-antigen-adjuvant conjugate in the footpad of mice, preferential uptake by APCs was mediated by the exposed antigen derived peptide and its CpG nucleic acids rather than by the APC-specific Ab [120]. Also when NPs were decorated with mannosylated alginate or different DC specific targeting Abs, this decoration was less influential on murine DC specific particle uptake, respectively [121, 122]. Furthermore, similar observations were made by our own group with "naked" mRNA where it was shown that after its intranodal delivery mainly CD8α^+ DCs were involved in its uptake [52]. This form of "passive" APC targeting was further evidenced by our own observation that noninfectious LVs were able to induce a similar therapeutic benefit in the E.G7-OVA tumor model as the APC targeted LVs after their intranodal delivery [45]. The latter was explained by the uptake of protein contaminants present within the noninfectious LV preparations, which were presumably taken up by the APCs in a nontargeted fashion. Notably, we did demonstrate that our APC targeted LVs outperformed the noninfectious LVs in terms of $CD4^+$ and $CD8^+$ T-cell stimulation, suggesting that the "active" APC targeting factor does account for better DC activation than "passive" uptake of noninfectious LVs. In contrast, when DC receptor internalization parameters were investigated as well as their impact on antigen presentation outcomes, targeting did turn out to be responsible for antigen presentation after Ab targeted vaccination *in vivo*. By analyzing endocytosis of DEC205, CLEC9A, CD11c, CD11b, and CD40 *in vitro*, they showed that neither the receptor expression level, speed of receptor internalization, and proportion of surface turnover nor the antigen load had an impact on MHC-I or -II mediated antigen presentation. On the contrary, $CD8^+$ or $CD8^-$ DC targeting did enhance MHC-II or -I mediated antigen presentation, respectively. Therefore, they concluded that receptor expression levels, speed of internalization, and/or the amount of antigen delivered could be excluded as major determinants of antigen presentation efficiency in the setting of Ab targeted vaccination [123]. One elegant approach is where receptors are upregulated prior to their targeting. This was investigated with a CD206 targeting cancer vaccine composed of mAb fused to an oncofetal protein. They showed that humoral responses to low vaccine doses could be enhanced by prior administration of GM-CSF, which upregulated CD206 expression in human mannose receptor transgenic mice, while coadministration of TLR agonists was required to elicit T_H1 immunity [124]. However, by prior administration of GM-CSF, one could question if it is the CD206 expression or the overall amount of APCs that is enhanced *in situ*. From these studies we can conclude that active APC targeting is still debatable and that more studies are warranted to unravel this enigma.

From a practical point of view it has been hypothesized that APC targeted delivery of vaccines could reduce their dose requirement. Hereby one has to distinguish two concepts: (1) APC specific targeting that results in an increased uptake of the vaccine in the case of otherwise "naked" molecules and (2) APC specific targeting to detarget vaccine delivery from all non-APCs in the case of infectious agents such as viral vectors. When APC specific uptake is enhanced, this generally results in a drastically reduced dose requirement [125, 126]. On the contrary, when we evaluated the CTL inducing capacity of broad tropism versus DC targeted LVs encoding OVA, we could not demonstrate a substantial benefit of DC targeted LVs over broad tropism LVs in terms of dose requirement without loss of efficacy [45]. Another striking observation was made by comparing different doses of *in situ* delivered targeted versus untargeted infectious nonreplicative OVA-encoding adenoviral vectors. While targeted delivery outperformed untargeted delivery after low dose administration, more effector $CD8^+$ T cells were induced with high doses of untargeted vaccine compared to targeted delivery. Interestingly, the protective capacity of the nontargeted vaccine was superior to that of the targeted vaccine in a tumor challenge model, demonstrating dose-dependent effects of DC targeting on the quality of the induced immune response [127].

In terms of safety, vaccine detargeting from nonimmunogenic stromal cells could reduce the risk of adverse reactions such as the development of autoimmunity and the induction of tolerance or unwanted systemic cytokine release due to overstimulation [119, 128, 129]. Indeed, targeted delivery of TLR agonists reduced their dose requirement by 100-fold and was associated with a decreased serum cytokine storm and related toxicities *in vivo*, compared to administration of soluble adjuvants [130]. Furthermore, APC targeting potentially reduces the risk for insertional mutagenesis when DNA and LV-based vaccines are directly administered since APCs are differentiated short-living cells which are unlikely to transform into malignant cells.

Next, APC subtype specific targeting is believed to allow the induction of a very specific fine-tuned immune response. Since the disentanglement of the heterogeneity of different APC subtypes, their specific targeting paved the way towards fundamental research on the exact therapeutic role of each APC subset in antitumor immunotherapy. Moreover, DC subtype specific targeting has already been reported for CLEC9A/BDCA-3 (murine/human cDC1), DCIR-2 (murine cDC2), BDCA-2 (human pDCs), SiglecH and BST2 (murine pDCs), and langerin (LCs) and showed promising differences in the elicited immune responses as a reflection of the specific function of these DC subtypes *in situ* [11, 81, 131–135]. However, DC subset specific functions are not fixed but vary

among several factors such as species and inflammatory state. This is exemplified by the presumed most favorable target for antitumor vaccination: the most professionalized cross-presenting XCR1/CLEC9A$^+$ cDC1 subset [88]. While they are peculiarly equipped to cross-present antigens from dead cells, they seem equally potent to cross-present soluble antigens when compared to other DC subtypes [136]. Of note, in mice the cDC1 subset represents the main IL-12 producing population, while, in humans, IL-12 production is not limited to the CD141$^+$ subset. Importantly, the lymphoid tissue CD8α^+ and nonlymphoid tissue CD103$^+$ DCs are also mediators of systemic and intestinal tolerance, respectively. Thus, the cDC1 lineage responds to its local microenvironment in order to induce either tolerance or cross-presentation dependent CD8$^+$ T-cell immunity. In line with these observations, the human BDCA3$^+$ DC equivalents of the murine DC8α^+ cDCs have also been shown to excellently cross-present antigens on the one hand but to suppress an immune response on the other hand by secreting IL-10 and inducing Tregs [137]. Nevertheless, when CLEC9A and XCR1, specific for cDC1, were targeted, this approach appeared potent to eradicate established melanomas [88, 138]. Interestingly, however, it was also shown that when CLEC9A was coupled to polyI:C, curdlan or nothing, the vaccine was able to modulate CD4$^+$ T cells into T_H1, T_H17 or Tregs, respectively [139], suggesting that the embedded adjuvant in the vaccine is more decisive for the immunological outcome than the cell type specific receptor towards which it is targeted.

Finally, targeting is believed to enhance the vaccine's immune stimulatory potential since detargeting TAAs from non-APCs but also B cells and macrophages could avoid the induction of tolerance or rapid antigen degradation. Indeed, when mice were treated with OVA, coupled or fused to Abs against DEC205, a more than 100-fold efficient and potent response was measured compared to untargeted antigens [128, 131]. Furthermore, several preclinical and clinical trials have demonstrated the effectiveness of APC targeted vaccines for human immunotherapy, which are summarized elsewhere [140]. However, most studies evaluating targeted antitumor vaccination are based on targeting receptors such as DEC205 and the mannose receptor CD206 (see Table 2), which are not specifically expressed by one particular DC subset. Therefore, it is hard to draw any conclusions with regard to an enhanced efficiency for antitumor immunity upon exclusive targeting of one DC subset. Moreover, when we targeted cDCs alone or also pDCs and macrophages using nanobody displaying LVs encoding OVA, we only observed clear differences in the induced CD4$^+$ T-cell profiles, while the therapeutic outcome of the cDC and the cDC as well as pDC targeted vaccine was comparable but most importantly less strong than that of the broad tropism LV vaccine [45]. Furthermore, it has been questioned if targeting as such is responsible for the increased immunogenicity compared to untargeted delivery, since this increase has also been ascribed to the immunomodulating role of the targeting moiety itself and less by targeting the specific DC subset [120, 121, 141].

So, based on our current knowledge, there is no strong rationale to target one DC subset over another to prime TAA specific CTLs and additional *in vivo* studies with human DC subset specific targets are definitely needed to identify the most specialized DC subsets, if any [142], a rationale that is further signified by reports on bystander maturation of cDCs by pDCs as well as on the need for multiple DC subset activation for optimal T_H1 and effector T-cell immunity [6, 14, 143]. This is exemplified by a study where the combination of BDCA3 and DC-SIGN targeted NPs was superior to targeting either subset alone in terms of T-cell activation. The mechanism underlying the observed synergy involved IL-15-dependent DC-DC cross talk suggesting that targeting only one APC subset could deprive the resultant immune response from the benefit of cross talk between different DC subsets [144]. Therefore, upcoming treatment paradigms should aim to include several primary DC subsets in a single vaccine as preclinical studies identified synergistic effects between various APCs [145].

5. Conclusions

An overload of targeted vaccination studies demonstrate that vaccination can be tailor-made to induce a particular phenotype of adaptive immunity by specifically targeting different surface molecules on DC subsets [146]. Nonetheless, conflicting results regarding the outcome of targeted vaccines to induce therapeutic antitumor immunity also stress that the benefit of targeting as such may not be overestimated. The immunogenicity of every vaccine, irrespective of its targeting abilities, is also characterized by its dose, size, surface charge, cargo, presence of adjuvants, route of administration, and the species to which it is delivered. So the question remains: does one targetable and omnipotent DC subtype really exists to increase the efficiency of current antitumor vaccination strategies?

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this review.

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