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TUMOR CELL/DENDRITIC CELL INTERACTIONS AND THE INFLUENCE OF TUMORS ON DENDRITIC CELL-MEDIATED ANTI-TUMOR IMMUNE RESPONSES AND DENDRITIC CELL-BASED TUMOR IMMUNOTHERAPIES

Topic Editors
Kristian Michael Hargadon
and Timothy Bullock

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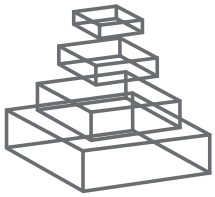

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TUMOR CELL/DENDRITIC CELL INTERACTIONS AND THE INFLUENCE OF TUMORS ON DENDRITIC CELL-MEDIATED ANTI-TUMOR IMMUNE RESPONSES AND DENDRITIC CELL-BASED TUMOR IMMUNOTHERAPIES

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Significant efforts over the last two decades have been made to better understand the factors that control DC maturation and activation and the impact of these processes on overall host immunity. In addition to the well-characterized role of DC in the induction of immunity to pathogens, a role for these cells as critical regulators of anti-tumor immune responses has more recently become apparent. These findings have generated interest in understanding how tumor/DC interactions impact the quality of anti-tumor immune responses, and they have contributed to increased enthusiasm for a variety of DC-based cancer immunotherapies. Such strategies have included DNA- or peptide-based vaccines that involve uptake and processing of tumor antigens by endogenous DC in cancer patients or the administration of tumor antigen-loaded exogenous DC-based vaccines. Additionally, many adjuvant, cytokine, and monoclonal antibody therapies aim either to enhance the immunostimulatory capacity of endogenous DC or to supplement the activity of these cells by targeting costimulatory receptors on T cells. Despite the promise of such therapeutic approaches for cancer treatment, their success is often limited, and much remains to be understood about how tumors influence DC function and the quality of DC-mediated immune responses. Tumor/DC interactions have therefore become an increasingly active area of investigation, and many studies have described effects of tumors on DC phenotype and function that include an accumulation of immature DC within tumors, tumor-altered differentiation of DC precursors into myeloid-derived suppressor cells, and the generation of tumor-associated DC with immunoregulatory properties. As this field moves forward, it will be important to gain mechanistic insights into the basis for both tumor-mediated DC dysfunction as well as the induction of either suboptimal or immunosuppressive adaptive anti-tumor immune responses by tumor-associated DC. Progress in these areas of tumor immunology will greatly improve our understanding of the factors that contribute to effective DC-mediated

anti-tumor immune control versus DC-associated anti-tumor immune dysfunction and subsequent tumor immune escape. Such information is vital for improving current and developing novel immunotherapeutic strategies for interfering with tumor-associated DC dysfunction and enhancing the functional quality of endogenous DC in cancer patients as well as the efficacy of exogenous DC-based anti-tumor vaccines. The articles contained within this special issue highlight these important topics and bring focus not only to our current understanding of tumor/DC interactions but also to major areas of investigation that remain ongoing in this field.

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EDITORIAL
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The role of tumor/dendritic cell interactions in the regulation of anti-tumor immunity: the good, the bad, and the ugly

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Since the discovery of dendritic cells (DCs) by Ralph Steinman and Zanvil Cohn 40 years ago (1), the role of these cells as critical regulators of immune tolerance versus activation has emerged as one of the most fundamental concepts in the field of immunology. Serving as a link between the innate and adaptive immune systems, DCs exhibit sensitive immune surveillance capabilities that enable their acquisition of antigens from a variety of sources in peripheral tissues, and they possess unique sensory properties and antigen processing machinery that enable their transformation into potent antigen-presenting cells (APCs). Importantly, the outcome (immune tolerance versus activation) of antigen presentation to T cells by DC is dependent on the maturation and activation state of the DC, and significant efforts over the last 20+ years have therefore focused on understanding factors that regulate DC maturation and activation. While their role in self-tolerance and the activation of T cell immunity to foreign pathogens has long been appreciated, more recently DCs have also been shown to play important roles in the regulation of anti-tumor immune responses. Since this time, considerable efforts have been placed on understanding many facets of tumor-associated DC, including: the induction, regulation, and maintenance of anti-tumor immunity by DC; tumor-associated interference with these processes to subvert anti-tumor immunity; and the application of this knowledge to develop therapeutic strategies for improving DC-mediated anti-tumor immune responses. In this collection of articles, we highlight our current understanding of the role played by DC in anti-tumor immunity and focus attention on important questions that remain to be answered in the field as we aim to improve the immunogenicity of tumor-associated DC and the outcome of DC-mediated anti-tumor immune responses in the future.

We begin this research topic with an Opinion article by Rolf Zinkernagel (2) and a responding Commentary from Anne Hosmalin (3), who offer opposing views on cross-presentation of tumor antigen by DC that we believe will generate interesting and thoughtful discussion. These articles are followed by a contribution from Schiavoni et al. (4) reviewing the major subsets of DC that have been implicated in cross-presentation and the role of type I IFN in enhancing DC-mediated cross-priming of anti-tumor CD8+ T cell responses. Research topic co-editor Kristian Hargadon then reviews the various levels at which tumor cells, tumor-derived factors, and tumor-associated cells in the milieu of the tumor microenvironment can interfere with DC function (5). Mechanistic insights into tumor-altered differentiation of DC precursors, tumor-associated suppression of DC maturation and activation, and tumor-induced development of regulatory DC with immunosuppressive function are highlighted, as are recent immunotherapeutic strategies that have been designed to prevent or overcome tumor-associated DC dysfunction and enhance the quality of anti-tumor immune responses. Co-editor Timothy Bullock further examines the metabolic changes that occur in DC during their maturation and discusses how dysregulated metabolism, particularly at the level of glycolysis and fatty acid metabolism, in tumor-associated DC may also impede maturation and contribute to the diminished immune stimulatory function of these cells (6). The impact of tumors on DC maturation is also explored by Dudek et al. (7), who describe the complexity of DC maturation status in the context of tumors, where the typical dichotomy of immature versus mature DC that regulate immune tolerance versus activation against clearly "self" or "non-self" antigen is less obvious. The authors describe a continuum of DC maturation states reported in the context of tumors that include not only the classical immature, tolerogenic DC and mature, immunogenic DC but also semi-mature DC which express low or even moderate levels of costimulatory molecules but which produce minimal stimulatory cytokines and therefore potentiate either tolerogenic or pro-tumorigenic responses. Studies that have identified factors (cytokines/chemokines, cell death modalities, and cancer cell-derived danger signals) regulating tumor-associated DC function are highlighted, as is the ability of anti-cancer therapeutic agents to influence and modulate the maturation states of DC. Additional discussion of this topic is provided by Ott and Bhardwaj (8), who speculate how tumor cell death resulting from MAPK pathway inhibition might enhance cross-presentation by DC in $BRAF^{V600}$ mutant melanoma patients, and by Palombo et al. (9), who describe various danger-associated molecular patterns (DAMPs) released during immunogenic cancer cell death that stimulate inflammatory DC to activate tumor-specific CD8+ T cell responses. This latter Perspective article

also discusses evidence for chemotherapy-associated induction of immune responses, particularly against antigens derived from proteins involved in stress pathways that are normally sequestered in healthy cells and therefore are not typically processed or presented to T cells. The authors suggest that such tumor-specific T cells are likely to be useful tools for identifying novel immunogenic tumor-specific antigens as these T cells can be isolated and "interrogated" with purified tumor proteins to assess which antigens are associated with high responsiveness. As researchers consider how cancer cell death influences immune responses in patients, and with renewed interest in the potential of combining traditional cancer therapy and immunotherapy (once thought to be mutually exclusive approaches to cancer treatment), these articles highlight the need to better understand how DC respond (particularly at the level of cytokine secretion) following cancer therapies that induce tumor cell death. Such knowledge will elucidate whether these approaches induce immunogenic versus tolerogenic cell death and promote development of semi-mature versus mature DC, and these insights will have significant implications for optimizing strategies to promote robust anti-tumor immune activation.

A recurring theme in many of the articles presented herein is that tumor immune evasion arises not only from a simple failure of the tumor microenvironment to support DC maturation but also from an active recruitment and exploitation by tumors of immature, tolerogenic DC that suppress adaptive responses. Seliger and Massa (10) review mechanisms by which tumor-derived soluble and membrane-bound factors alter myeloid and plasmacytoid DC function, including effects of these molecules on antigen processing and presentation by DC, T cell stimulatory capacity of DC, migration of DC to tumor-draining lymph nodes, and DC survival. Tesone et al. (11) discuss how tumor-altered myelopoiesis shifts differentiation of myeloid precursors from a DC-committed lineage to lineages with immunosuppressive functions such as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages, and these authors also focus on suppressive mechanisms that prevent DC maturation or that induce a switch from immunostimulatory to regulatory DC during tumor progression. Emphasis is also placed on specific recruitment of regulatory DC to tumors by tumor- and stroma-derived chemoattractants and how these tumor-infiltrating DC contribute to the overall suppressive nature of the tumor microenvironment. Vasaturo et al. (12) highlight specifically the negative immune regulation exhibited by PD-1, CTLA-4, and other co-inhibitory molecules and their receptors expressed on cells in the tumor microenvironment, including regulatory DC. These authors bring to light how such interactions hamper not only the induction of anti-tumor immunity by tumor-associated, tolerogenic DC that migrate to draining lymph nodes but also the effector activity of T cells that may have been activated appropriately in secondary lymphoid organs but whose effector function is subject to negative regulation following infiltration of tumors expressing co-inhibitory molecules. While the authors discuss the potential of manipulating costimulatory and co-inhibitory molecule expression in tumors and associated cells as a means of shifting the milieu of the tumor microenvironment from an immunosuppressive state to an immunostimulatory one, they also address the potential limitations of non-specifically administering immune checkpoint inhibitors that may result in autoimmune activation, thus underscoring the need to better understand ways of fine-tuning immune regulation by these molecules and of targeting them in a cell-specific fashion.

Because of the potential of DC to serve as both targets of and delivery agents for tumor immunotherapies, significant efforts have been focused on how best to utilize these cells in the treatment of cancer. Gallois and Bhardwaj (13) review mechanisms by which tumors, Tregs, and immunosuppressive myeloid cells impair DC function and discuss how interventions that aim to combat the suppressive tumor microenvironment can improve the clinical benefit of therapies involving *ex vivo*-generated DC or *in vivo*-targeted DC. In addition to highlighting the need to better understand tumor microenvironmental factors that should be targeted to improve the efficacy of DC-associated immune stimulation in cancer patients, the authors identify a variety of other factors that must further be studied to optimize these therapies, including mechanisms of antigen delivery to endogenous DC; the frequency, route, and site of DC vaccination; methods of DC activation both *ex vivo* and *in vivo*; and the particular DC subsets that should be employed or targeted during immunization. These and other factors are considered more specifically in the context of DC-based therapies in acute and chronic myeloid leukemia patients by Schürch et al. (14), in ovarian cancer patients by Goyne and Cannon (15), and in metastatic melanoma patients by van de Ven et al. (16). Additionally, Ott and Bhardwaj (8) offer insights into the impact of MAPK pathway inhibition on DC activation in melanoma patients carrying the $BRAF^{V600}$ mutation. Finally, Toubai et al. (17) review the roles of both host and donor DC in the induction of graft-versus-host disease and graft-versus-tumor effect following allogeneic hematopoietic stem cell transplantation, and the authors discuss strategies for, and challenges to, uncoupling these two processes as a means of maximizing anti-tumor immunity while minimizing autoimmune reactivity. It is clear from the work summarized in these articles that while this field has moved rapidly in recent years, much remains to be learned to optimize DC-related immune therapies for cancer. Moving forward, it is likely that combinatorial approaches that aim both to block immune inhibitory pathways and to promote immune stimulation will ultimately offer the greatest promise for successful DC-based cancer therapies. For instance, while the success of checkpoint blockade therapy has generally been limited to situations where T cells are already infiltrating tumors, therapies that also target DC, either endogenously or via vaccination, will likely promote anti-tumor T cell activation and therefore increase the proportion of patients for which checkpoint blockade is a viable option.

We conclude this collection of articles with a review by Chmielewski et al. (18), who describe MHC- and APC-independent immunotherapy using chimeric antigen receptor (CAR)-redirected T cells as an alternative to DC-based therapies and traditional adoptive T cell transfer therapy. In light of the tumor-associated suppression of DC described herein and the requirement for adequate expression of MHC molecules by both DC and tumor cells to achieve successful anti-tumor immunity, CAR-redirected T cell therapy has the potential to offer unique

advantages over traditional immunotherapies by allowing: (1) the targeting of not only peptides but also carbohydrates and inorganic compounds expressed on tumor cells and (2) the inclusion of intracellular costimulatory molecule signaling domains in chimeric receptors that overcomes the limitation inherent in conventional T cell recognition of tumor cells or tumor-altered DC that typically lack or express low levels of these molecules needed for T cell activation. Furthermore, although CAR-redirected T cells bypass the need for stimulation by DC, it is interesting to speculate that in addition to their direct anti-tumor activity, these T cells might also be useful for the licensing of DC in the tumor microenvironment, thereby indirectly leading to more robust endogenous anti-tumor immune responses as well.

As the intricacies of DC biology and the influence of tumors on DC phenotype and function continue to be uncovered, additional insights into the role of these cells in the induction, regulation, and maintenance of anti-tumor immune responses will continue to shed light on mechanisms of tumor immune escape and inform the design of novel therapies to enhance anti-tumor immunity. It is our hope that the advances highlighted herein and the questions raised for future consideration will generate additional discussion, drive experimental inquiry, and bring into focus the significance of tumor/DC interactions and their impact on overall anti-tumor immunity. Such emphasis is sure to bring rapid advancements in this field and will ultimately lead to the development of more effective cancer immunotherapies and improved clinical outcome in cancer patients in the future.

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OPINION ARTICLE
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On the role of dendritic cells versus other cells in inducing protective CD8+ T cell responses

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Dendritic cells (and/or macrophages) are key transporters of antigen from extra-lymphatic tissue to secondary lymphatic organs. The phagocytized antigen is presented via MHC class II but not via class I, except for infections by intracellular viruses, bacteria, etc. (1–4).

Class II-negative cells (e.g., fibroblasts) that get drained to secondary lymphatic organs (including spleen) induce MHC class I restricted CD8 T cells' cell responses as efficiently as dendritic cells (5–7).

So called crosspresentation is at least 10^5 times less efficient than direct presentation and therefore is practically not achievable under physiological conditions (5–8).

If antigen accumulates in the endoplasmic (ER) reticulum because of transport problems, crosspresentation on to MHC class I can be demonstrated. This requires gigantic amounts of antigen accumulation in the ER, but this process has so far been difficult to quantitate in comparison to direct presentation (9).

Positive demonstration of crosspresentation in experiments is sometimes based on use of excessive amounts of protein antigen (e.g., OVA) and/or the use of unphysiological (i.e., much too sensitive) detection method, e.g., using very high frequencies of transgenic T cells (e.g., OVA-specific tgCD8$^+$ T cells). In some experiments, virus inactivation is not controlled properly, permitting abortive (but not virus productive) infections that seemingly suggest crosspresentation instead of direct presentation [e.g., Ref. (8)].

An insulin-producing allogeneic cell graft strictly transplanted under the kidney capsule is accepted for more than >200 days by the host, but is promptly rejected if at the time of transplantation, or a few days later, the same cells are also given i.p. or i.v. (10) Once accepted, the allogeneic strictly peripheral cell graft is highly resistant to rejection by a transplanted corresponding allogeneic skin graft (or dendritic cells). This skin graft is rejected in a primary fashion, signaling absence of direct or indirect priming by the original allogeneic cell graft indicating absence of priming by the original cell graft. This prompt skin rejection does not cause rejection of the insulin-producing cell graft (10).

A strictly extralymphatic (7) tumor expressing a very strong and defined viral antigen (similar to insulin-producing self-beta-cells or allogeneic islet cells (10–12) can grow successfully to become lethal tumors. This depends on the condition that at the time of syngeneic tumor cell transplantation no (or too few) tumor cells escape/or drain to secondary lymphatic organs (7). This potentially early direct immunization is distinct from the late process of metastasis to secondary lymphatic organs that very often represent immune escape of tumor cells (e.g., MHC mutants, mutations of the T cell epitope, barrier formation by fibrin, coagulation, etc.)

DISCUSSION

DC transport antigen best to secondary lymphatic organs but only in an MHC class II associated fashion except of course if the DC is productively or abortively infected. The localization in or strictly outside of secondary lymphatic organs determines if and whether a CD8$^+$ T cell immune response is induced or not. Crosspresentation of antigen to MHC class I by DC or macrophages is an experimental artifact due to overdosage or uncontrolled new cell internal synthesis. Pure crosspresentation is so inefficient, that it is largely impractical for application and therapeutic use against solid peripheral tumors.

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GENERAL COMMENTARY
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Mining the resource of cross-presentation

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A commentary on

On the role of dendritic cells versus other cells in inducing protective CD8+ T cell responses
by Zinkernagel RM (2014) Front Immunol 5:30. doi:10.3389/fimmu.2014.00030

(1) In the face of MHC-class I-restricted direct presentation from a live, replicating viral infection – after the groundbreaking discovery of the MHC restriction of T cell responses by Rolph Zinkernagel and Peter Doherty (1) – cross-presentation is indeed a weaker phenomenon (2, 3). Antigen presenting cells can cross-present exogenous antigens from viruses which cannot infect them, allowing anti-viral MHC-class I-restricted, $CD8^+$, cytotoxic T cell priming (4). Nevertheless, when direct presentation is available, cross-presentation is dispensable for eliciting a maximal anti-vaccinia virus $CD8^+$ T cell response (5).

(2) Demonstration of cross-priming often uses sensitive detection methods requiring the infusion of high numbers of T cell receptor-transgenic mouse T cells (3). However, in many studies, natural CD8 T cell responses to epitopic peptides were induced in mice by cross-priming (6–11).

(3) Dendritic cells (DC) and/or macrophages are indeed key transporters of antigens to secondary lymphoid organs (12–16).

(4) Via class I, DC present phagocytosed antigens from intracellular viruses, bacteria, and other microorganisms, but also from non-replicating microorganisms [HIV inactivated by antiprotease, replication level proven unable to induce direct presentation (17)], apoptotic cells or tumor cell-derived fragments (9), or even live cells [DC purified after culture with live tumor cells, then injected, and the wash-out from these cells, containing potentially contaminating antigen from these tumor cells, is not able to present directly (18)]. This can lead to protective vaccination against tumors (18–20).

(5) Location in secondary lymphoid organs or tertiary lymphatic tissues may indeed be the key to CD8 T cell priming. Any cell type may be able to prime CD8 T lymphocytes when located in lymphatic tissues and correctly activated (21–23). This in turn requires appropriate draining of these cells into lymphatic tissues to provide antigen amounts high enough for cross-presentation, in the presence of the appropriate costimulation and cytokines to induce either immune responses or active tolerance. This conjunction of circumstances may be obtained less rarely with DC than with other cell types, thanks to their high expression of class I molecules, costimulation molecules and cytokines, and their high propensity to transport antigens to lymphatic tissues. This can yield the direct presentation of endogenous epitopes. Why would not it also yield the cross-presentation of exogenous antigens?

(6) During HIV infection, like in LCMV infection, chronic type I IFN production and immune hyperactivation induce immune suppression. Live replicating recombinant vaccine vectors that induce efficient direct presentation are not acceptable for immune therapy in populations with potential immune deficiency, for safety reasons. In addition, these vectors require the use of sequences, which will not mutate like the actual patient's viral sequences. Why not try and exploit this opportunity to stir the balance of HIV-specific immune responses toward immunity instead of tolerance?

(7) Tumors also favor suppressive mechanisms (negative costimulation molecules like CTLA-4 and PD-1, suppressive cytokines like IL-10, myeloid-derived suppressor cells). After tumor ablation, it may be hoped in the future to restore immune surveillance by anti-suppressive agents and therapeutic vaccination. Recombinant vaccines expressing tumor antigens for direct presentation require the use of sequences, which will not mutate like the actual patient's tumor antigen sequences. Antigens from dead tumor cells can be crosspresented, even though with a low efficiency, by DC, yielding protection against tumors *in vivo* (in experimental settings that are still artificial but protective) (9); this can be obtained more efficiently using live tumor cells (18). Why not try and exploit this opportunity to stir the balance of antitumoral immune responses toward immunity instead of tolerance?

(8) When major direct antigen presentation is not as blatant as during replicative viral infections, or is not exploitable for safety reasons, the alternative cross-presentation pathway may be exploitable for therapy.

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Type I interferons as stimulators of DC-mediated cross-priming: impact on anti-tumor response

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Induction of potent tumor-specific cytotoxic T-cell responses is a fundamental objective in anticancer therapeutic strategies. This event requires that antigen-presenting cells present tumor-associated antigens (Ag) on their MHC class-I molecule, in a process termed cross-presentation. Dendritic cells (DC) are particularly keen on this task and can induce the cross-priming of $CD8^+$ T cells, when exposed to danger or inflammatory signals that stimulate their activation. Type I interferons (IFN-I), a family of long-known immunostimulatory cytokines, have been proven to produce optimal activation signal for DC-induced cross-priming. Recent *in vitro* and *in vivo* evidences have suggested that IFN-I-stimulated cross-priming by DC against tumor-associated Ag is a key mechanism for cancer immunosurveillance and may be usefully exploited to boost anti-tumor $CD8^+$ T-cell responses. Here, we will review the cross-presentation properties of different DC subsets, with special focus on cell-associated and tumor Ag, and discuss how IFN-I can modify this function, with the aim of identifying more specific and effective strategies for improving anticancer responses.

Keywords: type I interferon, interferon alpha, cross-priming, dendritic cells, dendritic cell subsets, cancer, tumor-associated antigen, immunosurveillance

INTRODUCTION

Anti-tumor immune responses are evoked by several effector cells. These include both innate immune cells, like NK cells and macrophages, and cells of the adaptive immunity. Among these, $CD8^+$ T cells are ideal tumoricidals, due to their capacity to recognize and kill malignant cells in an antigen (Ag)-specific fashion and to establish a long-lasting protection. The activation of anti-tumor $CD8^+$ T-cell responses is fulfilled through a process known as cross-priming and requires the uptake of extracellular Ag also in the form of tumor cells by the antigen-presenting cell (APC), which subsequently delivers the engulfed material to a distinct endosomal/lysosomal pathway that allows the processed peptides to be presented on MHC class-I (MHC-I) molecules (cross-presentation) (1).

Among APC, dendritic cells (DC) have been described as the sole cell type able to cross-present cell-associated Ag and studies on both mouse and human models have revealed that distinct DC subsets display differential capacities to perform this process resulting in the induction of immunity or tolerance. In this respect, for cross-presentation to result in cross-priming, three signals must be delivered by DC: (i) loading and cross-presentation of cell-derived Ag onto MHC-I, (ii) appropriate co-stimulation through membrane molecules, and (iii) secretion of pro-inflammatory cytokines. Among cytokines produced by DC and capable of triggering DC activation, type I interferons (IFN-I) have been shown to play a major role in promoting cross-priming against both soluble proteins and cell-associated Ag, such as Ag derived from tumor apoptotic cells.

Here we discuss the most recent advances in Ag cross-presentation properties by several types of DC and on the capacity of IFN-I to turn on $CD8^+$ T-cell cross-priming.

DC SUBSETS CAPABLE OF MEDIATING CROSS-PRIMING

MOUSE DC

In the murine immune system several DC subtypes have been characterized (2). The spleen contains at least five subsets distinguished by expression of specific surface markers: plasmacytoid DC (pDC; $CD11c^{low}PDCA\text{-}1^+B220$), CD8α DC ($CD8\alpha^+CD4^-CD11b^-$), CD11b DC ($CD8\alpha^-CD4^-CD11b^+$), CD4 DC ($CD8\alpha^-CD4^+CD11b^+$), and merocytic DC (mcDC; $CD8\alpha^-CD4^-CD11b^-$). These DC subsets markedly differ in their abilities to capture and cross-present antigenic material and only some of them can cross-present cell-associated Ag (3).

CD8α DC is the most efficient DC subset in Ag cross-presentation uniquely able to prime $CD8^+$ T cells against cell-associated Ag *in vivo* (4–6). In the steady-state, CD8α DC capture dead cells resulting from constitutive turnover and play a central role in self-tolerance (6, 7). The *in vivo* relevance of CD8α DC in $CD8^+$ T-cell cross-priming against cell-associated Ag has been better clarified by studies with mice devoid of this DC subset. Mice deficient for either transcription factors Batf3 or NFIL3/E4BP4, both lacking CD8α DC selectively, display impaired cross-priming of $CD8^+$ T cells against cell-associated Ag (8, 9). Similarly, IRF-$8^{-/-}$ mice, which are devoid of CD8α DC and pDC, display impaired capacity to cross-present both soluble and tumor cell-derived Ag (10, 11).

Initial studies showing that CD8α DC capture cellular Ag more efficiently than other DC subsets suggested that this was the principal mechanism for increased cross-presentation ability by CD8α DC (5, 12). Indeed, CD8α DC selectively express some receptors, such as CLEC9A or Tim-3, involved in the recognition of necrotic and apoptotic cells, respectively, and implicated in cross-presentation of cellular Ag (13–15). Additional studies unraveled

that CD8α DC also possess a special processing machinery that delivers the internalized Ag onto the MHC-I processing pathway (16). Such machinery involves the activity of the small GTPase Rac2, selectively operating in CD8α DC, and the subcellular assembly of the NADPH oxidase complex (NOX2) to phagosomes that maintains a high phagosomal pH and thus facilitates cross-presentation (17).

The lymph nodes (LN) contain additional DC subtypes, termed migratory DC, arising from non-lymphoid tissues where they normally reside. Of the two types of migratory DC described, namely $CD103^{-}CD11b^{+}$ and $CD103^{+}CD11b^{-}$, only the latter has been described to cross-present cellular Ag captured either in the lung (18) or in the skin (19). The shared efficiency for Ag cross-presentation by CD8α DC and $CD103^{+}$ DC has been attributed to a developmental relationship, since these two DC subsets have a common dependence on the transcription factors Batf3, Flt3L, Id2, and IRF8 for their differentiation (20–22). Recent findings showed that CD8α DC and $CD103^{+}$ DC specifically co-express XCR1, a receptor for $CD8^{+}$ T-cell-secreted XCL1 that couples DC cross-presentation to induction of $CD8^{+}$ T-cell immunity (23, 24). XCR1 was found to be a conserved specific marker also for additional murine DC subtypes (including a small percentage of mcDC and of $CD103^{-}$ DC) and for human DC subsets devoted to cross-presentation of cell-associated Ag (25–27).

Janssen's group reported that mcDC capture dying cells, although less efficiently than CD8α DC, and cross-prime $CD8^{+}$ T cells for an extended time due to prolonged Ag storage (3, 28). *In vivo*, mcDC induce tumor-specific CTL responses in B16 melanoma-bearing mice (28). Of note, injection of tumor vaccine-loaded mcDC, but not of CD8α DC, elicited protective responses from subsequent tumor challenge in mice in a vaccination EL-4 thymoma model and resulted in therapeutic eradication of established EL-4 and B16 melanoma tumors (28, 29).

Although cross-presentation of soluble proteins by mouse pDC can occur upon Toll-like receptor (TLR) engagement (30), there is no evidence that pDC may cross-present cell-associated Ag. Instead, pDC can indirectly enhance $CD8^{+}$ T-cell cross-priming, through production of IFN-I and other soluble mediators (31–34). The capacity of CD11b DC to cross-present cellular Ag is also weak. In a murine model of mesothelioma expressing influenza virus hemagglutinin, as a membrane-bound neo-tumor Ag, one group has reported that both CD8α DC and CD11b DC from tumor-draining LN could cross-present membrane hemagglutinin (35). This observation suggests that the anatomical location may affect the efficacy of CD11b DC for tumor Ag cross-presentation.

HUMAN DC

Human DC also display some heterogeneity. In the blood, DC may be essentially distinguished into $BDCA1^{+}$ myeloid DC (mDC), $BDCA3^{+}$ mDC, and pDC. $BDCA3^{+}$ mDCs have been reported to cross-present Ag on their MHC-I molecules more efficiently than other DC populations. Due to functional and phylogenetic similarities, this subset is thought to be the human equivalent of mouse CD8α DC (36–38). $BDCA3^{+}$ mDCs selectively express CLEC9A and XCR1 and efficiently cross-present Ag derived from dead cells (25, 36, 37).

The role of human pDC as professional APC in the cross-presentation of exogenous Ag is under intensive investigation. Tumor cells infected with a measles virus vaccine are able to induce tumor Ag cross-presentation by human pDC via production of large amounts of IFN-α (39). Furthermore, harnessing uptake receptors to deliver Ag to pDCs can enhance cross-presentation and IFN-I production, resulting in the generation of potent anti-tumor responses (40). The efficacy of pDC has been verified in a cohort of metastatic melanoma patients in whom activated pDC were found to induce Ag-specific T-cell responses and significantly extended overall survival (41). It has been recently shown that pDC cross-present soluble and cell-associated tumor Ag to cytotoxic T lymphocytes to the same degree as $BDCA3^{+}$ mDC (42, 43). Indeed, two recent reports argued on the notion that cross-presentation is restricted to certain human DC subsets. Amigorena's laboratory showed that freshly isolated tonsil-resident pDC, $BDCA1^{+}$, and $BDCA3^{+}$ mDC cross-present soluble Ag with the same efficiency, displaying comparable phagosomal pH, production of reactive oxygen species and capacity to export internalized proteins to the cytosol (44). Delamarre's group reported that the diverse human DC subsets are equally able to cross-present exogenous Ag to $CD8^{+}$ T cells provided that the Ag is delivered to early endocytic compartments (43). These findings have extensive implications for vaccination strategies aiming at exploiting *ex vivo*-differentiated autologous DC, resembling primary DC subsets and endowed with strong cross-priming ability.

ENHANCEMENT OF CROSS-PRESENTATION BY IFN-I IN MURINE DC

In the steady-state or in the context of a tumor, DC cross-presentation of cell-associated Ag rarely results in $CD8^{+}$ T-cell cross-priming due to lack of immunostimulatory signals capable of activating DC. IFN-I is the prototype inflammatory cytokine released upon infection or under physiological distress acting as a stimulus for DC cross-priming (45). *In vivo*, IFN-I induces $CD8^{+}$ T-cell cross-priming against viral or soluble protein Ag through DC stimulation (46). Recently, we showed that IFN-I can affect DC cross-presentation of cell-associated Ag. *In vitro* or *in vivo* exposure of CD8α DC that have engulfed irradiated tumor cells to IFN-I resulted in three distinct effects: (i) increased retention of engulfed apoptotic material that correlated with decreased endosomal acidification and resulted in enhanced Ag cross-presentation, (ii) prolonged survival of phagocytic CD8α DC, and (iii) phenotypic activation of the cross-presenting DC that resulted in DC "licensing" for cross-priming (10). Similar results were obtained using tumor cells killed by the chemotherapeutic agent cyclophosphamide as a source of antigenic material for CD8α DC. In this setting, addition of IFN-I resulted in $CD8^{+}$ T-cell cross-priming *in vitro* and tumor rejection *in vivo* (47).

Two different groups have recently reported the *in vivo* relevance of endogenous IFN-I signaling on CD8α DC for promoting $CD8^{+}$ T-cell-dependent spontaneous tumor rejection. Diamond et al. (48) showed that mice lacking IFN-α/β receptor 1 selectively in DC cannot reject methylcholanthrene-induced fibrosarcoma, a highly immunogenic tumor normally rejected by immunocompetent mice, and that CD8α DC from these mice display

defective Ag cross-presentation to $CD8^+$ T cells. Similarly, by using IFN-α/β receptor $1^{-/-}$ and $Batf3^{-/-}$ mice transplanted with B16 melanoma, Fuertes et al. (49) reported that endogenous IFN-I, produced shortly after tumor challenge, was essential for intratumoral accumulation of CD8α DC and for induction of tumor Ag-specific T-cell priming and tumor rejection via CD8α DC stimulation. These studies underscore CD8α DC as fundamental targets for endogenous IFN-I-mediated spontaneous immune control of a rising tumor.

Cross-priming mediated by mcDC also requires IFN-I. However, unlike CD8α DC, which fail to produce IFN-I upon uptake of apoptotic cells, mcDC are able to do so. Adoptive transfer experiments revealed that this endogenous IFN-I acts in an autocrine manner to activate mcDC and is both necessary and sufficient for boosting $CD8^+$ T-cell cross-priming against cell-associated Ag (28, 29). Of interest, endogenous IFN-I signaling in mcDC was essential for preserving internalized material from early degradation and endosomal acidification similarly to what observed with CD8α DC exposed to exogenous IFN-I (3, 10). These findings suggest that IFN-I promote cross-priming in DC by exploiting a mechanism involving regulation of endosomal pH and Ag retention that direct the antigenic cargo toward the MHC-I processing pathway, as also observed with human DC (see below) (50). Thus, regulation of phagosomal acidification may be viewed as a strategy exploited by inflammatory signals, such as IFN-I, to switch on cross-priming in those DC subsets that under steady-state are devoted to tolerance induction and may provide a mechanism (coupled to MHC-I upregulation) by which IFN-I induce autoimmune reactions, namely by enhancing presentation of self Ag.

The ability of some compounds targeting TLR to stimulate $CD8^+$ T-cell cross-priming has also been shown to occur through endogenous IFN-I production and subsequent DC stimulation (51, 52). The efficacy of CpG in cancer immunotherapy is dependent on cross-talk between pDC and conventional DC (mcDC and CD8α DC), the first serving as a source of IFN-I through TLR9 triggering and the latter responding to IFN-I to promote $CD8^+$ T-cell cross-priming and anti-tumor response in melanoma-bearing mice exposed to cryoablation (53).

ENHANCEMENT OF CROSS-PRESENTATION BY IFN-I IN HUMAN DC

Type I interferons exert multiple effects on human DC, affecting the major cellular pathways associated to their APC function, namely differentiation, maturation, and migration (54, 55). Human immature conventional DC treated *in vitro* with IFN-I upregulate the expression of MHC-I, CD40, CD80, CD86, and CD83 molecules resulting in a superior capacity to induce $CD8^+$ T-cell responses (56, 57). Moreover, IFN-I support the differentiation of human monocytes into DC with high capacity for Ag presentation (58). IFNα induces one-step differentiation of human monocytes into highly activated and partially mature DC (IFNα-DC), retaining a marked phagocytic activity and exhibiting a special aptitude for inducing $CD8^+$ T-cell responses (59, 60). Studies on phenotype and functions of IFNα-DC have pointed that these cells can resemble naturally occurring DC, generated from monocytes in response to danger signals, including infections when high levels of IFN-I are released (61–65). Indeed, subtypes of DC resembling IFNα-DC have been observed in patients suffering from autoimmune or infectious diseases (54).

IFNα-DC express markers involved in antigen processing such as CD208 and the scavenger receptor oxidized low-density lipoprotein receptor 1 (LOX-1), implicated in Ag uptake and $CD8^+$ T-cell cross-priming (66). *In vivo*, IFNα-DC generate cytotoxic responses and $CD8^+$ T-cell cross-priming against viral and tumor-associated Ag (59, 67–69). Efficient cross-presentation of tumor-associated Ag by IFNα-DC loaded with apoptotic human melanoma cells was found to correlate with enhanced proteasome activity (68). In addition, studies employing soluble Ag point to an effect of IFNα in preserving Ag from early degradation, thus facilitating its routing onto MHC-I pathway (50). Thus, although the intracellular mechanisms underlying the superior efficiency of IFNα-DC in Ag cross-presentation need to be clarified, these evidences suggest that IFN-I may control this process at diverse levels.

Interestingly, IFNα-DC have been reported to exhibit some phenotypic features of pDC (70). We recently reported that IFNα-DC and pDC share a similar miRNA signature as well as some phenotypic and molecular markers potentially accounting for common functional activities, such as IFN-I production upon viral infection. Moreover, IFN-I was also able to affect some functions of pDC, including the expression of the pDC-associated markers IRF-8 and TLR-9 (71).

IMPORTANCE OF DC CROSS-PRIMING FOR ANTICANCER IMMUNE RESPONSES AND PERSPECTIVES FOR EXPLOITATION OF IFN-I POTENTIATING EFFECT

Several lines of evidence indicate that DC-mediated cross-priming is crucial for anti-tumor immunity (72). First, tumor-infiltrating DC purified from tumor samples have the capacity to cross-present tumor Ag *in vitro* (73). Second, priming *in vivo* of anti-tumor T-cell responses can be abrogated in models in which DC subsets specialized for cross-presentation can be specifically depleted. Indeed, $Batf3^{-/-}$ mice are unable to reject highly immunogenic tumors due to defective cross-presentation by $Batf3^{-/-}$ DC, reduced tumor-infiltration of $CD8^+$ T cells and failure to develop tumor-specific CTL (8).

The therapeutic anti-tumor potential of IFN-I has been appreciated since 1960s (74, 75). However, only recently it has become clearer how IFN-I participate in naturally occurring, protective immune responses to primary tumors, thus playing a prominent role in cancer immunosurveillance. In addition, IFN-I has been shown to be a crucial component of cancer-immunoediting, namely the process whereby the immune system suppresses cancer growth and shapes tumor immunogenicity (76, 77). These findings have renewed the interest in exploiting the anti-tumor potential of IFN-I in therapeutic and vaccination strategies against cancer.

Therapeutic approaches that involve either exogenous IFN-I administration or its induction within the tumor microenvironment have shown effects on $CD8^+$ T-cell responses via DC stimulation at various levels. In mice with established B16 tumors, radiotherapy induced a local increase in IFN-I expression by myeloid immune infiltrates that acted enhancing the cross-priming ability of tumor-infiltrating DC and was crucial for host therapeutic response (78). Furthermore, intratumoral delivery of IFN-I synergized with immunotherapy (79) and chemotherapy (47) to

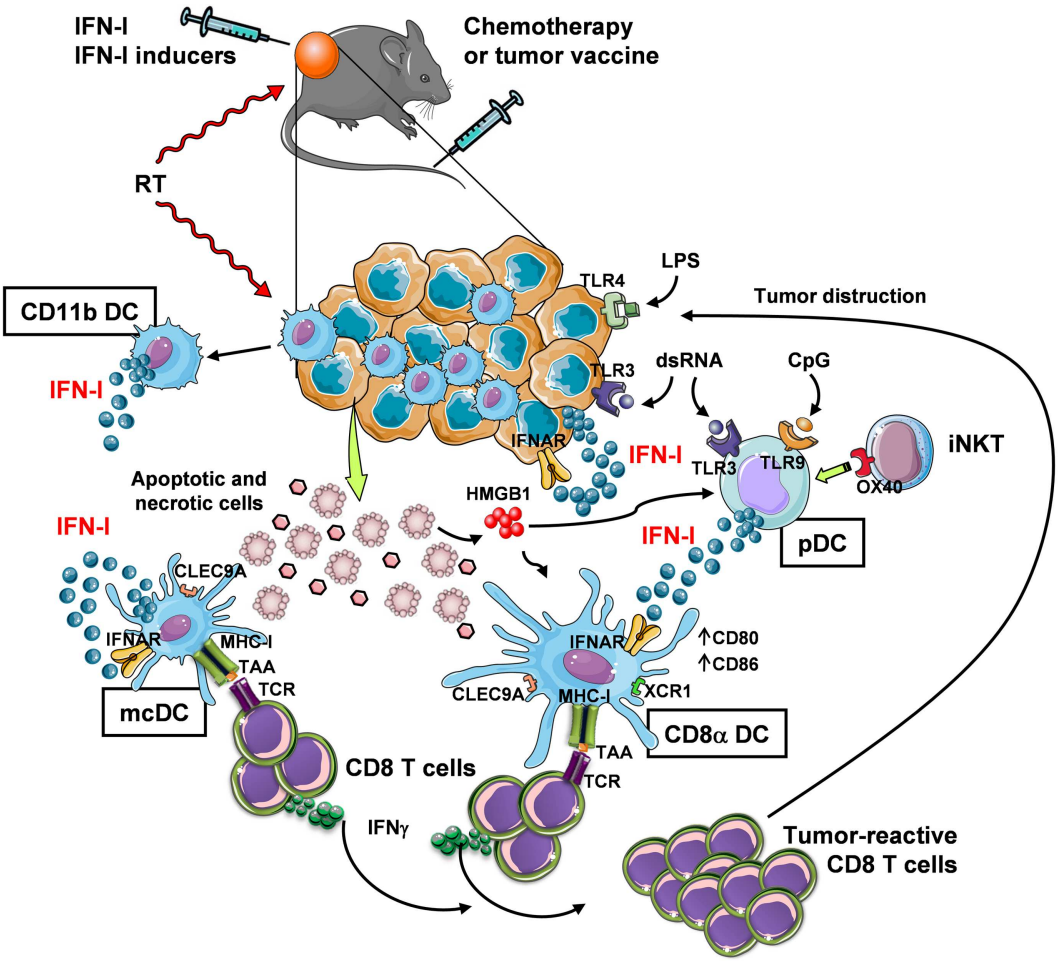

FIGURE 1 | *In vivo* induction of anti-tumor CD8$^+$ T-cell responses through IFN-I-mediated DC cross-priming at the tumor site. Systemic chemotherapy and local radiotherapy (RT) induce tumor cell death that result in the availability of antigenic material (which is otherwise provided by a tumor vaccine) for internalization by specialized DC subsets, namely mcDC and CD8α DC. These subsets then cross-present the tumor-associated Ag (TAA) through their MHC-I complex to CD8$^+$ T cells. In order to induce CD8$^+$ T-cell cross-priming, the cross-presenting DC need to be exposed to activation stimuli (DC licensing), such as IFN-I. While mcDC spontaneously produce IFN-I that act in an autocrine fashion to induce DC licensing for cross-priming, CD8α DC are unable to do so and require the exogenous cytokine. Thus, in the tumor site IFN-I may be made available in different manners: (1) via intratumoral injection, (2) by RT, which stimulates IFN-I release by infiltrating myeloid CD11b DC (and possibly other immune and non-immune cells), and (3) by intratumoral delivery of IFN-I-inducing substances, such as TLR ligands. Some TLR ligands can also bind to tumor cells that express TLR3 and TLR4 to trigger autocrine IFN-I production and stimulation of DC. Alternatively, TLR ligands, such as dsRNA and CpG, stimulate pDC to produce large amounts of IFN-I. IFN-I secretion by pDC may also be stimulated by invariant NKT (iNKT) cells via OX40 and HMGB1 released by dying tumor cells. The final outcome of these events is the expansion of tumor-reactive CD8 T cells with killing activity.

induce therapeutic response in tumor-bearing mice that involved, in both cases, enhanced DC cross-presentation. Notably, IFN-I can enhance anti-tumor CTL responses also via direct effects on CD8 T cells, inducing their expansion and acquisition of effector functions thus improving therapeutic efficacy (80, 81).

With regard to protocols employing vaccine preparations, co-administration of CpG with a DC vaccine was found to overcome tumor-specific tolerance after stem cell transplantation, inducing protective anti-tumor response through CpG-induced IFN-I *in vivo* (82). Recently, Shimizu and colleagues showed that vaccination with B16 melanoma cells loaded with the invariant NKT cell ligand αGalCer stimulated tumor-reactive CD8$^+$ memory T cells in a novel mechanism involving cross-talk between XCR1-expressing DC and pDC via NKT-stimulated IFN-α production by pDC (33). Human studies also point to the use of IFN-I-inducers as promising approach to boost anti-tumor effector responses. The efficacy of topical application of the TLR7/8 agonist imiquimod, the only TLR agonist approved by FDA for skin cancer treatment, has been linked to local increase of IFN-I production, recruitment of DC and induction of tumor-reactive CTL (83). Finally, it is worth mentioning that tumor-derived IFN-I may also positively contribute to anti-tumor immune response. In virtue of their TLR expression, B16 melanoma cells were found to respond to ligands to TLR3 and TLR4 by releasing substantial levels of IFN-I that

induced DC activation and resulted in tumor growth inhibition by the host (84, 85).

CONCLUDING REMARKS

Despite IFN-α has received approval for therapy of several neoplastic diseases, side effects of systemic long-term treatments and insufficiently high efficacy have challenged its use in current clinical protocols. Therefore, novel strategies to exploit IFN-I in therapeutic and vaccination protocols are needed that take into account, for example, controlled timing of administration to avoid negative feedback mechanisms in the responding immune cells (58, 86), and the involvement of active cross-talk between multiple types of immune cells that play different, non-overlapping roles within the tumor site. In this view, the combined use of chemotherapy or radiotherapy that kill cancer cells, providing source of Ag for DC, with exogenous IFN-I or compounds capable of inducing IFN-I *in situ* may be viewed as promising strategies for boosting DC cross-presentation and CTL induction within the tumor microenvironment (**Figure 1**).

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Tumor-altered dendritic cell function: implications for anti-tumor immunity

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Dendritic cells (DC) are key regulators of both innate and adaptive immunity, and the array of immunoregulatory functions exhibited by these cells is dictated by their differentiation, maturation, and activation status. Although a major role for these cells in the induction of immunity to pathogens has long been appreciated, data accumulated over the last several years has demonstrated that DC are also critical regulators of anti-tumor immune responses. However, despite the potential for stimulation of robust anti-tumor immunity by DC, tumor-altered DC function has been observed in many cancer patients and tumor-bearing animals and is often associated with tumor immune escape. Such dysfunction has significant implications for both the induction of natural anti-tumor immune responses as well as the efficacy of immunotherapeutic strategies that target endogenous DC *in situ* or that employ exogenous DC as part of anti-cancer immunization maneuvers. In this review, the major types of tumor-altered DC function will be described, with emphasis on recent insights into the mechanistic bases for the inhibition of DC differentiation from hematopoietic precursors, the altered programing of DC precursors to differentiate into myeloid-derived suppressor cells or tumor-associated macrophages, the suppression of DC maturation and activation, and the induction of immunoregulatory DC by tumors, tumor-derived factors, and tumor-associated cells within the milieu of the tumor microenvironment. The impact of these tumor-altered cells on the quality of the overall anti-tumor immune response will also be discussed. Finally, this review will also highlight questions concerning tumor-altered DC function that remain unanswered, and it will address factors that have limited advances in the study of this phenomenon in order to focus future research efforts in the field on identifying strategies for interfering with tumor-associated DC dysfunction and improving DC-mediated anti-tumor immunity.

Keywords: dendritic cell, tumor, differentiation, maturation, activation, immunosuppression, tumor microenvironment, immunotherapy

INTRODUCTION

Dendritic cells (DC) are critical regulators of host immune responses that serve as a bridge between innate and adaptive immunity. Following their differentiation from either myeloid or lymphoid bone marrow-derived progenitors, DC populate both lymphoid and peripheral tissues, where they are involved in immune surveillance and control of immune reactivity in the host. DC precursors may differentiate into a variety of specialized subsets that exhibit numerous immunoregulatory activities, and the diverse functions of these cells are tightly linked to their maturation and activation status (1). Immature DC are highly phagocytic and function to sample both soluble and cell-associated antigens (Ag) in host tissues. In the steady state, such immature DC either fail to elicit immune responsiveness to Ag they have acquired (2), or they actively induce immune tolerance to these Ag (3–6). On the other hand, stimulation of immature DC by a variety of factors [including pathogen-associated molecular patterns, danger-associated molecular patterns (DAMPs), inflammatory mediators, CD40L, etc.] induces the maturation and activation of these cells, thereby converting DC into potent stimulators of immune activation. Such DC upregulate expression of costimulatory molecules, cytokines, and chemokines necessary for the activation and recruitment of T lymphocytes and other immune effectors into a response to eliminate the source of Ag representing danger to the host (7–9).

In addition to their role in activation of immunity against foreign pathogens, DC have also been shown to be critical players in the induction of anti-tumor immune responses (10–12). The role of DC in eliciting such responses is highlighted by studies demonstrating immunologic ignorance of tumors under conditions in which cross-presentation of tumor Ag by DC is precluded (13–17). However, despite the ability of DC to elicit tumor Ag-specific T lymphocyte responses, in many cases these responses are dysfunctional and ineffective in clearing the tumor (18–24). While such immune dysfunction might result from direct suppression of T cells by tumors or tumor-derived factors, it may also arise indirectly from suboptimal stimulation or suppression of T cells by tumor-altered DC. Tumor-altered DC function has now been documented in many cancer patients and tumor-bearing animals and ranges from influences of tumors on the differentiation of

DC from hematopoietic precursors to effects on the behavior of fully differentiated DC. These effects on DC and their precursors can lead to accumulation in the tumor microenvironment of a variety of cells that include myeloid-derived suppressor cells (MDSC), tumor-associated macrophages (TAM), immature DC, and immunoregulatory myeloid DC (mDC) and plasmacytoid DC (pDC), each of which exhibit distinct phenotypic characteristics (**Table 1**). The identification of such cells in cancer patients not only has important prognostic value, but it also has significant implications for (1) the induction of natural anti-tumor immune responses and (2) the efficacy of immunotherapeutic strategies that target endogenous DC *in situ* or that employ exogenous DC as part of immunization maneuvers. Therefore, because of the importance of DC differentiation, maturation, and activation in dictating the immune stimulatory versus inhibitory activities of these cells, interference with any of these processes by factors or cells within the tumor microenvironment may greatly influence the induction and maintenance of anti-tumor immune responses in the host. This review will summarize the current knowledge regarding tumor-altered DC function and its impact on anti-tumor immunity, and it will highlight both recent advances in the field as well as important questions that will need to be answered as efforts are made to improve the quality of DC-mediated anti-tumor immune responses and DC-based cancer immunotherapies in the future.

Table 1 | Phenotypic characteristics of tumor-associated DC and populations derived from DC precursors.

Cell population	Cell surface markers	Soluble proteins
Immature DC	CD11c^{high}, CD80$^{-/low}$, CD86$^{-/low}$, MHC class I/II^{low}	
Mature/activated mDC	CD11c^{high}, CD80^{high}, CD83, CD86^{high}, MHC class I/II^{high}	IL-12p70
MDSC	CD11b, Gr-1 (mice) CD11b, CD14$^{-/+}$, CD15, CD33, MHC class II$^{-/low}$ (humans)	Arginase I iNOS ROS IDO
TAM (M2-like)	CD11b, CD14, CD68, CD115, CD163, CD204, CD301, CD312, F4/80	VEGF HIF TGFβ IL-10 Arginase I ROS
Regulatory mDC	CD11c^{high}, CD40^{low}, MHC class II^{low}, B7-H1^{high}, B7-DC^{high}	Arginase I IL-10 TGFβ
pDC	CD11c$^{low/int}$, CD19, B220/CD45R, BDCA-4, MHC classII^{low}	IFNα
Regulatory pDC	CD11c$^{low/int}$, CD19, B220/CD45R, BDCA-4, MHC classII^{low}, ICOS-L	IDO

TUMOR-ALTERED DIFFERENTIATION OF DC PRECURSORS AND ACCUMULATION OF MYELOID-DERIVED SUPPRESSOR CELLS AND TUMOR-ASSOCIATED MACROPHAGES WITHIN TUMORS

Dendritic cells are specialized cells that differentiate from both myeloid and lymphoid progenitors before acquiring their unique functions as Ag presenting cells (APC), and a number of studies have described factors derived from both tumors and associated cells within the tumor microenvironment that interfere with DC differentiation from precursors, thereby contributing to a loss of stimulatory APC activity in tumor-bearing hosts. Soluble factors secreted by human renal cell carcinomas and pancreatic cancers, including IL-6 and M-CSF, have been shown to block DC differentiation from CD34$^+$ progenitors and promote lineage commitment toward CD14$^+$ monocytes that express little to no MHC and costimulatory molecules and that fail to induce allogeneic T cell proliferation in mixed leukocyte reaction (MLR) assays (25, 26). Similar inhibition of CD34$^+$ precursor cell differentiation into DC has been attributed to tumor-derived VEGF (27), and this blockade of DC differentiation is associated with suppression of NF-κB activity in these cells (28). VEGF has also been implicated in suppressing the differentiation of skin-resident Langerhans cells in a murine fibrosarcoma model (29). In addition to secreting cytokines that inhibit DC differentiation, tumors may also secrete other factors that interfere with the development of different subsets of DC. The gangliosides GD2 and GM3 secreted by human and murine neuroblastoma cell lines have been shown to inhibit differentiation of DC from CD34$^+$ progenitors (30), and human melanomas secrete GM3 and GD3 gangliosides that not only inhibit DC differentiation from monocytic precursors but also induce apoptosis of monocyte-derived DC (31, 32). Likewise, cyclooxygenase-1 (COX-1)- and COX-2-derived prostanoids present in primary tumor-derived supernatants from several freshly isolated human tumor types block DC differentiation as well (33), and the source of these suppressive mediators may be not only tumor cells themselves but also stromal cells within the tumor microenvironment, as stromal cell-derived prostaglandin-E_2 (PGE_2) was recently shown to inhibit the differentiation of both bone marrow- and monocyte-derived DC (34). Regardless of the mechanism of inhibition, the loss of APC function associated with suppressing DC differentiation may significantly limit the induction of anti-tumor immune responses and contribute greatly to tumor immune escape.

In addition to the inhibitory effects of tumor-derived and tumor-associated factors on DC differentiation that preclude the development of cells with APC function, there is an abundance of data documenting how these factors can also alter the differentiation program of DC precursors and promote the accumulation of immature myeloid cells with immunosuppressive function (**Table 2**). These MDSC, characterized by expression of CD11b and Gr-1 in mice and a number of cell surface markers in humans (**Table 1**), are associated with various cancer types and have been recovered at high levels from both tumors and tumor-draining lymph nodes (35–38). Their induction may be driven by a number of factors released by tumors and tumor-associated cells, including VEGF (39), TGFβ (40), IL-1β (41), IL-13 (42), GM-CSF (43), prostaglandins (44), reactive oxygen species (ROS) (45), and components of the complement system (46). Differentiation into

Table 2 | Mechanisms of immune suppression by MDSC.

Suppressive mediator	Cellular target	Impact on target
Reactive oxygen species	T lymphocytes	↓ IL-2, ↓ proliferation, ↑ apoptosis
IDO	$CD8^+$ T lymphocytes $CD4^+$ T lymphocytes	Anergy Induction of Tregs
Arginase I	T lymphocytes	↓ CD3ζ chain ↓ Proliferation Expansion of Tregs
TGFβ	$CD4^+$ T lymphocytes NK cells	Induction of Tregs Anergy ↓ NKG2D ↓ IFNγ ↓ Cytotoxicity
CCL3, CCL4, CCL5	$CD4^+$ Tregs	Recruitment to tumor
IL-10	Macrophages	↓ IL-12
???	DC	↓ Phagocytosis ↓ Maturation ↓ Migration ↓ T cell stimulation

???, unidentified factor(s).

MDSC is associated with hyperactivation of STAT3 (39, 47, 48) and is accompanied by acquisition of a number of immunosuppressive properties. In a murine sarcoma model, MDSC suppression of Ag-specific $CD8^+$ T cell responses required direct cell–cell contact via TCR/MHC class I and was mediated by release of ROS (49–51). Similarly, MDSC lines generated from mice bearing adenocarcinomas exhibit nitric oxide-mediated suppression of IL-2 signaling in activated T cells. In this model, nitric oxide production by MDSC required direct contact with, and IFNγ secretion by, the activated T cell, and this nitric oxide inhibited T cell proliferation and induced T cell apoptosis (52, 53). More recently, the increased production of ROS by MDSC was shown to be the result of upregulated NADPH oxidase activity in these cells in several different murine models and in head and neck cancer patients (54). MDSC have also been shown to induce T cell tolerance through release of indoleamine 2,3-dioxygenase (IDO) and arginase I, enzymes involved in degradation of tryptophan and arginine, respectively (55, 56). In regard to the latter, tumor-derived COX-2 can mediate PGE_2 signaling in MDSC, thereby triggering overexpression of arginase I in these cells (57). In both murine models and cancer patients, tumors are enriched in arginase I-producing MDSC, and arginine metabolism in the tumor microenvironment leads to downregulation of CD3ζ chain and suppression of proliferative capacity in both $CD4^+$ and $CD8^+$ T cells (58, 59). Interestingly, recent studies evaluating the abundance and activity of MDSC in tumors, tumor-draining lymph nodes, and peripheral blood of cancer patients have shown that both the frequency and arginase I activity of these cells correlates with the clinical stage of the tumor, thus suggesting a critical role for these immunosuppressive cells in disease progression (60, 61).

In addition to the direct tolerization of anti-tumor T lymphocyte responses by MDSC, these cells are also known to induce the development of regulatory T cells (Tregs) that can also suppress T cell activation. In this light, MDSC-derived TGFβ not only suppresses cytolytic activity of T lymphocytes (42), but it has also been demonstrated in the B16 murine melanoma model to promote expansion of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs in both tumors and tumor-draining lymph nodes (62). Others have reported that tumor-infiltrating MDSC isolated from B16 melanomas also express high levels of the chemokines CCL3, CCL4, and CCL5, the ligands for the CCR5 chemokine receptor that is preferentially expressed on Tregs (63). These results indicate that MDSC likely also play a critical role in recruitment of expanded Tregs into the tumor microenvironment. Additionally, TGFβ-independent MDSC induction of Tregs has been reported in a B cell lymphoma model, where expansion of preexisting natural Tregs required Ag presentation and arginase I activity by MDSC (64). A link between MDSC and Tregs has also recently been reported in a study of breast cancer patients, where the presence of IDO-expressing MDSC correlated with increased infiltration of Tregs into primary tumors and lymph node metastases (56).

The immunosuppressive activities of MDSC extend beyond regulatory effects on T lymphocytes as well. In a murine model of gestation-enhanced metastasis of B16 melanoma, MDSC diminished the number and activity of NK cells (65). Likewise, in both mammary carcinoma and hepatic tumor models, MDSC suppressed NKG2D expression, IFNγ secretion, and cytotoxic activity by NK cells (66, 67). In the hepatic tumor model, suppression required direct cell–cell contact and was mediated by membrane-bound TGFβ on MDSC, and this interaction caused NK cells to be hyporesponsive to activating stimuli, indicating that they had acquired an anergic phenotype. Similar findings have been reported in patients with hepatocellular carcinoma, where suppression of NK cell activity was dependent on MDSC engagement of the NKp30 receptor on NK cells (68). MDSC have also been shown both to impede the maturation and T cell stimulatory capacity of DC (69) and to engage in cross-talk with macrophages, leading to diminished IL-12 secretion by macrophages and increased IL-10 production by MDSC (70). Such alteration of cytokine secretion patterns has the potential to polarize helper and cytotoxic T cells toward a type 2 response that is less robust in its anti-tumor efficacy.

In addition to shifting the differentiation of myeloid precursors away from DC lineage commitment and promoting development of MDSC, tumors can also drive the differentiation of DC precursors into other immunosuppressive cells of myeloid origin, most notably TAM. Recently, these cells have been shown to descend from both bone marrow-derived and splenic precursors, and some populations are believed to reflect the culmination of MDSC differentiation (71). Importantly, accumulation of TAM, particularly those with an anti-inflammatory M2-like phenotype, correlates with poor prognosis in patients with a variety of cancers (72–76). T_H2 cytokines, glucocorticoids, and growth factors present in the tumor milieu are all known to induce M2-like macrophages (77), and tumor-derived IL-10 has specifically been shown to inhibit

DC differentiation from monocytic precursors and to promote the development of TAM from these cells (78). Much like MDSC, these TAM can suppress a variety of immune effectors and promote Treg suppressive functions through production of TGFβ, IL-10, and arginase I (38, 58, 79, 80). They have also been shown to induce T cell apoptosis by upregulating expression of B7-H1 (81), the ligand for the PD-1 receptor on T cells. Taken together, the diverse effects exerted by TAM and MDSC on cells of both the innate and adaptive immune systems contribute greatly to the immunosuppressive nature of the tumor microenvironment, and these phenomena highlight the role that tumor-altered differentiation of DC progenitors into MDSC and TAM plays in promoting tumor immune escape.

TUMOR-ASSOCIATED SUPPRESSION OF THE MATURATION AND ACTIVATION OF DIFFERENTIATED DC

In addition to subverting anti-tumor immunity by altering the differentiation of DC precursors and either preventing acquisition of APC function by these cells or inducing their development into immunosuppressive MDSC or TAM, tumors may also interfere with the maturation and activation of fully differentiated DC. While *in vitro* studies have shown that the release of heat-shock proteins by necrotic tumors triggers DAMP-mediated DC maturation (82, 83), and the presence of mature tumor-infiltrating DC correlates with the magnitude of anti-tumor T cell responses and disease prognosis in cancer patients (84, 85), a number of studies have described the accumulation of fully differentiated, yet immature, DC in tumors as well (86–88). Although a lack of mature DC in tumor tissue might reflect tumor-induced death of these cells (31, 32, 89), this phenomenon does not explain the accumulation of immature DC often seen in tumors. In cases where immature DC are recovered from tumors, it is often unclear whether the immature phenotype of these cells reflects a simple failure of tumors to support DC maturation and activation or, alternatively, an active suppression of DC maturation by tumors. One study demonstrated that administration of anti-CD40 Ab to tumor-bearing animals leads to maturation of DC capable of stimulating T cell activation (90), suggesting that the tumor either fails to support DC maturation or that suppression of DC maturation by the tumor is a reversible process. In support of the latter possibility, it has been shown that the maturation of tumor-infiltrating DC is enhanced following dissociation of DC from the tumor and overnight culture *ex vivo*, demonstrating that the tumor had actively limited DC maturation *in vivo* (91). Other studies have revealed that tumor-infiltrating DC are refractory to some maturation stimuli but not others, indicating that tumors can actively suppress DC maturation but that in some cases this suppression can be reversed under appropriate stimulatory conditions (92–94). Interestingly, in a comparative study of melanoma patients exhibiting either progressing or regressing metastases, DC isolated from patients with progressive disease expressed significantly lower levels of costimulatory molecules than those taken from patients with regressing tumors. Furthermore, DC from patients with regressing metastases induced robust T cell proliferation, while DC from patients with progressing metastases induced T cell anergy (95). Collectively, these data suggest that the context in which the tumor is encountered by DC is likely to impact the quality of their maturation, activation, and immunostimulatory capacity, and they emphasize the need to understand the role of tumor-derived factors and the tumor microenvironment in regulating the function of tumor-associated DC.

The limiting number of DC that can be isolated from tumor-bearing animals and cancer patients and the complex nature of the cell types and soluble proteins present within the tumor microenvironment have made it difficult to gain mechanistic insights into tumor-associated suppression of DC maturation *in vivo*. *Ex vivo* experiments with monocyte-derived and bone marrow-derived DC (BMDC) have been used as an alternative to *in vivo* studies for evaluating the suppression of DC maturation by tumor cells or tumor-conditioned media (96–98). Recent studies using these and similar *ex vivo* models have shown that interference with the HIF-1-induced COX-2/PGE_2 and VEGF pathways in colon cancer cells and knockdown of TGFβ expression in hepatocellular carcinoma both restore DC maturation that is otherwise suppressed by these tumors (99, 100). In another system involving a multicellular tumor spheroid three-dimensional model of melanoma, tumor-derived lactic acid was shown to suppress the production of several proinflammatory cytokines, including IL-12, by monocyte-derived DC and to limit the ability of these cells to induce T cell proliferation (101). Importantly, though, because these *ex vivo* systems often require differentiation of DC from progenitors in culture, it is often unclear from these studies whether the effects observed stem from a direct influence of tumors on DC or instead from an indirect action mediated by an influence of tumors on other cells in the culture that have not differentiated into DC. Therefore, to overcome the limitations inherent with studying the influence of tumors on DC function in both *in vivo* and *ex vivo* settings, DC lines that can be maintained as highly pure populations in culture have been generated and are a useful tool for *in vitro* studies aimed at understanding the basic biology of these cells (102–105). Such lines have enabled direct analyses of tumor/DC interactions, and it has recently been shown that melanoma-derived factors suppress the LPS-induced maturation of both the DC2.4 and JAWSII DC lines (106). In a related study, a comparative analysis of multiple murine melanoma cell lines demonstrated that the suppression of DC2.4 costimulatory molecule and proinflammatory cytokine/chemokine expression correlates with the tumorigenicity of the melanoma under study (107), with the highly tumorigenic B16 melanoma exhibiting significantly greater suppression than its poorly tumorigenic, chemically mutated variant D5.1G4. These findings again point to a potentially vital role for tumor/DC interactions in the regulation of overall anti-tumor immunity and tumor outgrowth. It will be interesting to evaluate differences in the profile of immunosuppressive mediators released by these particular melanoma cell lines, as this analysis will identify potential candidate molecules involved in the suppression of DC maturation and activation by this cancer. While concerns have been raised that maneuvers employed to immortalize DC lines may alter the maturation state of these cells and their responsiveness to regulatory factors, many of these lines do exhibit the characteristics of immature DC and are responsive to traditional maturation stimuli (108–110). Therefore, additional studies using these DC lines and other tumor

systems can offer proof-of-principle data that tumors interfere with DC maturation in a straightforward, cost-effective model, and such investigations will provide further mechanistic insight into tumor-associated suppression of DC maturation and activation. Furthermore, observations made in such *in vitro* systems are likely to inform the design of experiments evaluating the role of tumor-derived factors in the suppression of DC maturation and activation in more physiologically relevant *ex vivo* and *in vivo* settings. Collectively, use of these different models will increase our understanding of tumor-induced suppression of DC function, and these insights will suggest immunotherapeutic strategies designed to reverse or prevent this suppression and enhance the immunostimulatory capacity of tumor-associated DC.

TUMOR-ASSOCIATED INDUCTION OF IMMUNOSUPPRESSIVE REGULATORY DC

The suppression of DC maturation and activation by tumor cells or factors within the tumor microenvironment has significant implications for the induction of T cell immunity to tumors, as immature DC are poor APC and do not efficiently stimulate T cell activation. There is also now substantial evidence that tumors not only suppress DC maturation but that they can also induce the development of regulatory DC that actively display immunosuppressive activity themselves. In fact, recent studies have demonstrated that progression of ovarian cancer from an immunologically controlled state to metastatic disease is accompanied by a switch in the phenotype and function of tumor-associated DC. Whereas DC isolated from ascites or draining lymph nodes of early-stage tumor-bearing mice elicited robust T cell responses, those isolated from mice with advanced disease induced minimal T cell proliferation and suppressed T cell activation by immunocompetent DC (111, 112). Immunosuppressive DC isolated from late-stage tumor-bearing animals downregulated MHC class II and CD40 expression but significantly upregulated the co-inhibitory molecule B7-H1 and exhibited arginase I activity comparable to that seen in MDSC. These immunosuppressive activities were driven by tumor-derived PGE_2 and TGFβ (112). Other studies have also demonstrated tumor-induced upregulation of DC co-inhibitory molecules, including both B7-H1 and B7-DC (10, 96), as well as tumor-enhanced secretion of arginase I (113, 114) and TGFβ (115) by DC that inhibit T cell effector function and promote Treg development, respectively. In both tumor-bearing mice and prostate cancer patients, the expression of these and other immunoregulatory molecules by tumor-associated DC resulted from elevated expression of FOXO3 (116), a transcription factor recently shown to mitigate DC stimulatory capacity (117). Additionally, inhibition of T cell effector activity by tumor-associated regulatory DC has also been associated with increased IL-10 secretion by these cells. A variety of soluble factors present in colorectal tumor explant cultures, including VEGF and the chemokines CCL2, CXCL1, and CXCL5, were shown to enhance IL-10 production by DC (118, 119). Non-soluble mediators expressed on colorectal carcinoma cells can contribute to this process as well, as IL-10 production by DC was increased following engagement of DC-SIGN by tumor-associated cell surface glycans (120). Likewise, recombinant MUC1 mucins glycosylated in a manner equivalent to those expressed on breast carcinoma cells and natural MUC1 mucins in supernatants of human pancreatic carcinoma cell lines both suppress IL-12 production and promote IL-10 production by monocyte-derived DC, and these regulatory DC are poor stimulators of T cell proliferation and CTL activity but potent inducers of T cell anergy and $CD4^+$ Tregs (121, 122). IL-10 production by tumor-associated DC that inhibit anti-tumor T cell responses and promote tumor outgrowth has also been reported to be induced by COX-2/PGE_2 (123, 124). Similarly, in a murine myeloma model, tumor-derived IL-6, IL-10, and TGFβ were all shown to contribute to p38 MAPK signaling-mediated effects on BMDC maturation that led to decreased production of IL-12 and increased production of IL-10 by DC, and these cells elicited poor tumor-specific T_H1, CTL, and antibody responses (125). Hyperactivation of MAPK signaling similarly inhibited IL-12 production and T_H1 stimulation by melanoma-altered DC, though these effects were independent of IL-10, TGFβ, VEGF, and PGE_2 in tumor lysates (97). In addition to suppressing the development of T_H1-type immunity, other studies have shown that melanoma, as well as breast cancer, triggers DC-mediated induction of T_H2-like responses that promote tumor development (126, 127). Identification of factors produced by these tumors and their role in MAPK hyperactivation in DC will be crucial to developing strategies for skewing anti-tumor T cells toward type 1 responses that are more efficient in mediating tumor rejection.

In addition to the regulatory DC activities described above, which are largely associated with conventional mDC, a specialized subset of DC that develop immunosuppressive activity in the context of many tumors is the pDC. IDO-expressing pDC can be induced by tumor-derived PGE_2 (128) and have been recovered from tumor-draining lymph nodes of both melanoma-bearing animals and cancer patients (129). These cells suppress $CD8^+$ T cell responses to Ag presented by the pDC themselves as well as to those presented by third-party, non-suppressive APC. In addition to inducing $CD8^+$ T cell anergy, IDO production by pDC also promotes the differentiation of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs (130). Interestingly, pharmacologic blockade of IDO leads to enhanced IL-6 production by pDC that converts tolerogenic $CD4^+$ Tregs into T_H17-like cells, and this conversion correlates with enhanced $CD8^+$ T cell activation and anti-tumor immunity (131). $CD4^+$ Treg induction by pDC can also be mediated by engagement of ICOS on T cells with ICOS-L on pDC, and ICOS-L^+ pDC infiltration of tumors is associated with poor prognosis and disease progression in both breast and ovarian cancer patients (132–134). Tumors can also subvert immunity by regulating pDC production of IFN-α, a type I IFN that functions as a "signal 3" cytokine for $CD8^+$ T cell activation (135) and that promotes the survival and Ag retention of $CD8\alpha^+$ DC that cross-prime tumor-specific $CD8^+$ T cells (11). In clinical studies, tumor-associated pDC have been isolated by magnetic activated cell sorting via BDCA-4 positive selection of lineage-negative enriched mononuclear cells obtained from patient biopsies. In patients with aggressive breast cancers, these pDC exhibit suppressed IFN-α secretion and are able to sustain $CD4^+$ Treg expansion (136), and the suppression of IFN-α production by pDC has been attributed to tumor-derived TGFβ and TNFα mediated-signaling in these cells (137). Finally, pDC isolated from ascites of ovarian carcinoma patients have also been shown to induce $CD8^+$ Tregs that secrete high levels of IL-10 and suppress

T cell proliferation (138). Altogether, these findings demonstrate the complexity of the tumor microenvironment and its ability to induce a variety of immunoregulatory activities in DC that impact the function of multiple cell types involved in anti-tumor immune responses (**Table 3**). Tumor-associated conversion of these potentially immunostimulatory APC into suppressive cells is therefore a significant hurdle to the induction of effective anti-tumor immunity that contributes greatly to tumor immune evasion.

IMMUNOTHERAPEUTIC STRATEGIES FOR INTERFERING WITH TUMOR-ASSOCIATED DC DYSFUNCTION

The induction of DC dysfunction is a major impediment to the activation and maintenance of successful anti-tumor immunity (**Figure 1**). Indeed, in addition to its documented impacts on anti-tumor T cell responses summarized herein, this phenomenon may also explain the unaccounted for presence of dysfunctional T cells associated with naturally generated immune responses in other experimental animal models and cancer patients (18–24). Additionally, tumor-associated DC dysfunction may limit the efficacy of immunotherapeutic strategies that rely on the activity of DC *in situ* to stimulate anti-tumor immunity, and it may therefore explain the lack of success observed thus far with many DNA-, peptide-, and protein-based immunization maneuvers that require endogenous DC to process and present tumor Ag to specific T cells (139–143). Even the quality of responses elicited following immunization with previously activated, exogenous DC may be compromised by an influence of tumor-associated factors on DC function. Importantly, though, insights into the mechanistic bases for tumor-associated DC dysfunction have informed the design of novel DC-based cancer immunotherapies, and many of these strategies have enhanced the T cell stimulatory capacity of DC and led to induction of more robust and efficacious anti-tumor immune responses.

Several strategies have been employed to promote DC differentiation from hematopoietic precursors and prevent the accumulation and suppressive activities of tumor-associated cells of myeloid origin. For instance, both IL-4 and IL-13 were shown to prevent renal cell carcinoma-induced blockade of DC differentiation (144). Similarly, administration of the anti-VEGF Ab bevacizumab to patients with lung, breast, and colorectal carcinoma led to a decrease in the frequency of MDSC and enhanced the T cell stimulatory capacity of DC (145). Abrogation of MDSC immunosuppression can also be achieved by exposure of these cells to all-trans retinoic acid, which induces the differentiation of MDSC isolated from a number of murine tumors and renal cell carcinoma patients into mature immunostimulatory DC (146, 147). Others have demonstrated that interference with STAT3-mediated-signaling reverses immune suppression by MDSC and enables differentiation of these cells into mature DC (39, 61). One study also showed that interference with both STAT3 and p38 MAPK signaling pathways in monocyte progenitors further improved the quality of tumor-associated DC, blocking the inhibitory effects of tumor-derived factors on DC differentiation from these progenitors and skewing the IL-12/IL-10 cytokine profile of the resulting DC toward a T_H1-promoting phenotype (148). Based on these data, it is not surprising that vaccination with exogenous, STAT3-depleted DC was shown to enhance anti-tumor $CD8^+$ T cell responses and improve control of tumor outgrowth (149).

Table 3 | Induction and suppressive activity of tumor-associated regulatory DC.

Tumor-derived factor	Regulatory DC activity	Impact on host immunity
TGFβ, PGE_2	↓ MHC II ↓ CD40 ↑ B7-H1 ↑ Arginase I	↓ T cell proliferation ↓ T cell effector function
VEGF CCL2, CXCL1, CXCL5 Glycans COX-2/PGE_2	↑ IL-10	↓ T cell effector function
MUC1 mucins	↓ IL-12 ↑ IL-10	↓ T cell proliferation ↓ CTL activity T cell anergy ↑ $CD4^+$ Tregs
IL-6, IL-10, TGFβ	↓ IL-12 ↑ IL-10	↓ T_H1 polarization ↓ CTL activity ↓ Ab response
???	↑ TGFβ	↑ $CD4^+$ Tregs
PGE_2	↑ IDO by pDC ↓ IL-6 by pDC	$CD8^+$ T cell anergy ↑ $CD4^+$ Tregs
TGFβ, TNFα	↓ IFNα by pDC	↑ $CD4^+$ Tregs ↑ $CD8^+$ Tregs
???	↑ ICOS-L by pDC	↑ $CD4^+$ Tregs

???, unidentified factor(s).

In addition to strategies that interfere with the development and suppressive activities of tumor-associated myeloid cells, several approaches are being explored for improving the quality of fully differentiated DC in the context of tumors as well. *In vivo* administration of nanoparticles carrying immunostimulatory miRNA converts endogenous immunosuppressive DC into cells capable of activating robust anti-tumor responses that inhibit progression of established ovarian cancers (150). Moreover, supplementation of stimulatory cytokines whose expression is often suppressed in tumor-associated DC, such as IL-12 and IFNα, can enhance T cell effector function elicited by endogenous DC (151, 152). Significant efforts have also been made to optimize exogenous DC-based cancer immunotherapies. Several studies have investigated various maturation protocols for exogenous DC in order to best promote the immunostimulatory capacity and vaccine efficacy of these cells (153–156). One group has reported that treatment of PBMC-derived immature DC with various combinations of cytokines and inflammatory stimuli, namely LPS + IFNγ, LPS + IFNγ + IL-1β, and LPS + IFNγ + IL-1β + TNFα, results in no discernible difference in DC expression of costimulatory molecules or IL-12 (153). On the other hand, substantial differences in DC maturation have been observed following exposure of

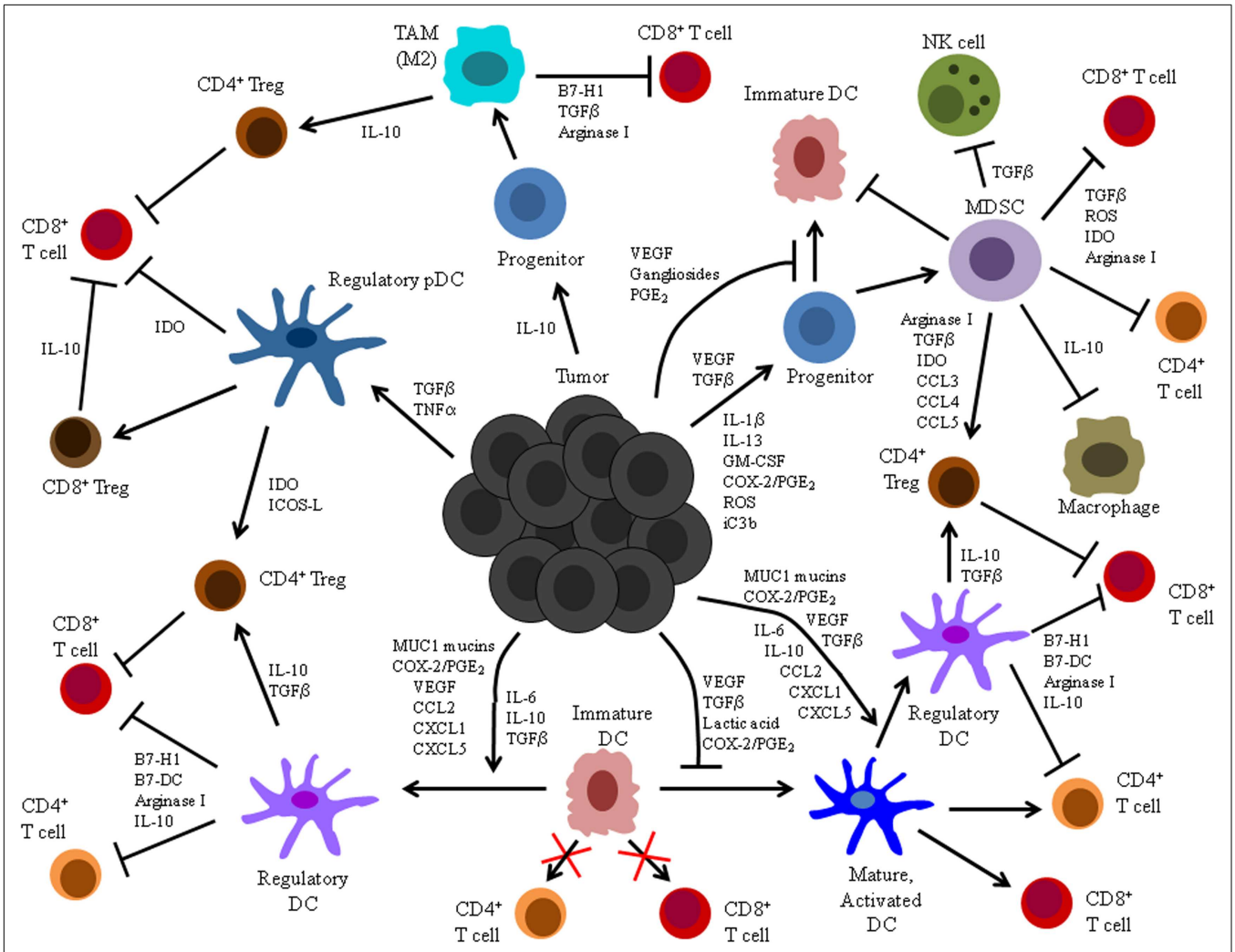

FIGURE 1 | Summary of tumor-altered DC function. Illustrated here are the mechanisms by which tumors alter DC function and the processes by which these altered cells impact host anti-tumor immunity. Tumors secrete a variety of factors that can: (1) inhibit differentiation of DC from precursors, (2) induce differentiation of DC precursors into immunosuppressive MDSC or TAM, (3) suppress maturation, activation, and stimulatory APC function of already differentiated DC, and (4) induce development of immunosuppressive regulatory DC.

immature DC to a mixture of various other inflammatory mediator/cytokine cocktails. Stimulation with lipid A and IFNγ resulted in significantly higher DC expression of costimulatory molecules and IL-12 than stimulation with a combination of TNF-α, IFN-α, IFN-γ, IL-1β, and poly(I:C) or a combination of TNF-α, IFN-γ, IL-1β, and CL097 (156). Still others have evaluated DC maturation following exposure to tumor lysates. PBMC-derived DC treated with lysates from heat-shocked melanoma cells exhibited robust maturation and immunostimulatory capacity, as these cells were capable of cross-presenting melanoma-associated Ag and inducing anti-tumor $CD8^+$ T cell responses (154). Importantly, heat-shocking of melanoma cells induced membrane translocation of CRT and expression of HMGB1, and the maturation of immunostimulatory DC in this study was dependent on their recognition of these tumor-derived "danger signals." It has also recently been shown that TNFα can augment tumor lysate-induced DC maturation (155). In addition to investigating strategies for optimal induction of DC maturation, many researchers have employed strategies to block the suppressive effects of tumor-derived factors on exogenous DC. In this light, DC genetically engineered to secrete a VEGF/vascular permeability factor decoy receptor that neutralizes soluble VEGF and precludes signaling in DC resulted in increased expression of costimulatory molecules and proinflammatory cytokines/chemokines by DC and improved CTL activity and anti-tumor immune control in a murine colon cancer model (157). Similar improvements in the efficacy of an exogenous DC vaccine were observed following neutralization of tumor-derived TGFβ (158). Alternatively, other approaches for enhancing exogenous DC-induced anti-tumor immune responses aim at blocking either the immunosuppressive mediators expressed by tumor-altered DC or the targets of these mediators expressed on other immune cells. In a murine model of breast cancer, siRNA-mediated silencing of IDO in vaccinating DC enhanced the ability of these cells to stimulate

T cell proliferation and CTL effector function, decreased the induction of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs, and led to enhanced control of tumor outgrowth (159). Similarly, immunization with IL-10-deficient DC conferred enhanced protective and therapeutic immunity against a murine hepatocellular carcinoma (160). Furthermore, DC genetically engineered to interfere with immunomodulatory receptors expressed on endogenous immune cells, such as CTLA-4 on effector T cells and GITR on Tregs, can enhance the overall immunogenicity of these cells as well (161, 162). Improved anti-tumor immunity has also been observed for a DC-based vaccine administered in combination with anti-CTLA-4 Ab and Treg-depleting anti-CD25 Ab (163). Likewise, administration of neutralizing Ab that interferes with the B7-H1/PD-1 pathway improved the efficacy of a DC/tumor fusion vaccine in multiple myeloma patients (164). Finally, it is also possible to improve the efficacy of both exogenous and endogenous DC-based vaccines by transducing DC either *ex vivo* or *in vivo* with viral vectors that encode immunostimulatory molecules. A number of studies have reported improved anti-tumor immunity when this approach was used to drive expression of CD80/CD86 costimulatory molecules (165, 166) or IL-12 (167) by DC. Collectively, these strategies highlight the advances made in tumor immunotherapy as our understanding of tumor immune suppression and evasion has evolved over the last several years. As additional insights into tumor-altered DC function are gained, optimization of these current immunotherapies and development of novel strategies for enhancing anti-tumor immune responses will further improve the efficacy of DC-based cancer vaccines.

CONCLUSION

Tumor immunosurveillance is now a well-documented phenomenon whereby host immune cells and effector molecules function to recognize and eradicate developing tumors in the body. At the heart of this process are DC, innate immune cells that function to acquire tumor Ag through phagocytosis, activate adaptive immunity against these specific tumor Ag, and recruit immune effectors to the site of the tumor for immunologic destruction of these transformed cells. However, one of the hallmarks of cancer growth is immune evasion, and tumor cells may evolve a number of escape mechanisms during their progression that subvert immunosurveillance. A significant contributor to tumor immune evasion is the alteration of DC function by tumors and associated factors present in the tumor microenvironment. As discussed, such alteration of DC function may include effects on the differentiation of DC from bone marrow-derived precursors, suppression of the maturation and activation of already differentiated DC, and the induction of immunosuppressive regulatory DC that inhibit anti-tumor immune responses. Over the last several years, significant efforts have been made to gain mechanistic insights into these processes of tumor-altered DC function. These findings have in turn led to the development of several immunotherapeutic strategies for improving the function of tumor-associated DC. Still, much remains to be learned about the processes by which tumors impact the function of DC and how such altered DC influence the quality of other immune effectors. As this field moves forward, it will be important to increase our understanding of factors that contribute to tumor recognition by DC and to identify additional tumor-associated DAMPs and inflammatory stimuli that promote optimal maturation and activation of immunostimulatory DC. Additionally, a better understanding of how tumor microenvironmental factors impact the quality of DC differentiation, maturation, and activation will suggest new possibilities for interfering with the suppression of these processes by tumors. Such knowledge will enable the optimization of current, and the development of novel, DC-based immunotherapies that aim to improve the quality and outcome of host anti-tumor immune responses.

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Metabolic influences that regulate dendritic cell function in tumors

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Dendritic cells (DC) are critical regulators of both activation and tolerance in the adaptive immune response. The dual nature of DC immunoregulatory function depends on their differentiation and activation status. DC found within the tumor microenvironment (TME) and tumor-draining lymph node often exist in an inactive state, which is thought to limit the adaptive immune response elicited by the growing tumor. The major determinants of DC activation and the functional alterations in DC that result from integrating exogenous stimuli have been well investigated. Extensive efforts have been made to elucidate how the TME contributes to the inactivated/dysfunctional phenotype of tumor-associated DC (TADC). Although performed predominantly on *in vitro* DC cultures, recent evidence indicates that DC undergo required, coordinated alterations in their metabolism upon activation, and dysregulated metabolism in TADC is associated with their reduced immunostimulatory capacity. In this review, we will focus on the role of glycolysis and fatty acid metabolism in DC activation and function and discuss how these metabolic pathways may be regulated in TADC. Further, we consider the need for developing novel experimental approaches to assess metabolic choices *in vivo*, and the necessity for integrating metabolic regulation into the optimized development of DC for tumor vaccines and immunotherapy for cancer.

Keywords: dendritic cell, tumor-associated dendritic cell, activation, metabolism, glycolysis, oxidative phosphorylation, lipid metabolism, cancer immunotherapy

INTRODUCTION

Dendritic cells (DC) serve as sentinels of the immune system. They constantly acquire antigen (Ag) from their environment and degrade it into short peptides that are presented at the cell surface in association with MHC molecules for surveillance by T cells. The inflammatory context in which DC exist influences their expression of critical co-stimulatory molecules and cytokines (**Figure 1B**) that provide the context for Ag presentation. Factors that promote the expression of co-stimulatory molecules and cytokines support the activation, expansion, and survival of responding T cells. In the absence of co-stimulation, DC present Ag in a manner that induces tolerance in the specific T cell repertoire, by mechanisms such as deletion (1) and anergy (2). During infection, inflammatory cytokines such as TNFα and type-1 interferons (IFN-1), or pathogen associated molecular patterns (PAMPs), induce a program of activation that initiates the CCR7-dependent migration of DC from the periphery to draining lymph nodes (3, 4). Additional stimulation via CD40 can further raise the activation state of DC, in part by inducing the expression of CD70 (**Figure 1B**) (5–7), leading to what is referred to as a licensed T cell response. While these basic tenets of DC activation have been well investigated, and extensively reviewed elsewhere (8, 9), recent studies have brought to light metabolic transitions in DC that are necessary for them to attain full function, or can regulate their functional activation. Here, we discuss the impact of these metabolic alterations on DC function; how metabolic pathways may be regulated in tumor-associated DC (TADC); and given the immature state of DC often found in tumors [and the negative prognosis associated with such immaturity (10, 11)] we consider the influence of the tumor microenvironment (TME) on these functions.

REQUIREMENT FOR GLYCOLYSIS AND DC ACTIVATION

Substantial evidence demonstrates that upon activation immune cells undergo a metabolic reprograming, switching from oxidative phosphorylation (OXPHOS) to aerobic glycolysis, a phenomenon initially observed in cancer cells in 1920s by Warburg (the Warburg effect) (12). In cancer cells, the Warburg effect is induced by growth factor signaling or by mutations in metabolism-related intrinsic pathways [such as loss-of-function mutants of succinate dehydrogenase (SDH) and Fumarate hydratase (FH), and constitutive activation of hypoxia-inducible factor-1 (HIF-1) and (c-Myc)] (13, 14), while T cells undergo the metabolic switch upon T cell receptor (TCR) activation by Ag in the context of proper co-stimulation (15). This change in cellular metabolic pathways provides essential metabolic and bio-energetic resources to support programs of new gene expression and protein synthesis during robust cellular proliferation. (16, 17)

A recent study from the Pearce group reported that PAMP stimulation of TLR induces a metabolic transition in resting immature DC, characterized by a conversion from mitochondrial β-oxidation of lipid and OXPHOS (**Figure 1A**) to aerobic glycolysis (**Figure 1B**) (18). Unlike in cancer cells and effector T cells, the Warburg effect in DC does not fuel cell division but rather appears to be crucial for DC activation and survival upon TLR stimulation.

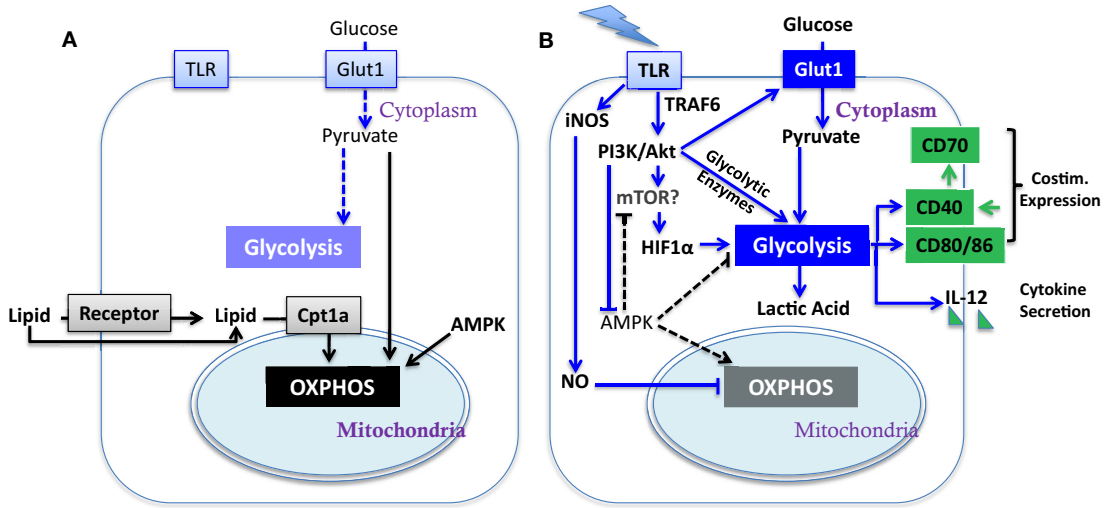

FIGURE 1 | Metabolic regulation of DC activation. Illustrated here are metabolic pathways in resting DC **(A)** and activated DC **(B)**. Upon TLR stimulation, DC undergo a metabolic switch from OXPHOS (key mediators are linked by black arrows) to glycolysis (key mediators are linked by blue arrows), which contributes to their activation (major determinants in green; costim. is short for co-stimulatory molecule). Block arrows mark blockade and dash lines show insignificant process. The impact of mTOR on glycolysis has yet to be fully elucidated in DC (see Section "AKT and mTOR in DC Function" in text).

During the early phase (within 5 h) after exposure to TLR agonists, absence of glucose in culture medium led to profound defects in DC activation, including surface expression of CD40 and CD86 and production of IL-12p40 (**Figure 1B**). Subsequently, DC activated by TLR signals are highly reliant on glucose for survival and become more sensitive to death by nutrient limitation (18). Thus, initiating glycolysis at the time of DC activation is critical for full activation independent from its role in subsequent survival (18). The glycolytic pathway, rather than OXPHOS, may be required due to the need to generate substrates that will be used during DC activation. Alternatively, components of the glycolytic pathway, such as GAPDH, can directly regulate protein translation and may be responsible for regulating the translation of proteins that are critical for DC activation. Further studies will be necessary to elucidate the mechanism by which glycolytic pathway promotes the DC maturation process.

The induction of glycolysis and DC maturation could be influenced by the tumor and the TME at several salient junctures (**Figure 2**). First, in the context of tumors, it is unclear whether the "find-me, eat-me" signals generated by damage associated molecular patterns (DAMPs)/alarmins, such as nucleotides, uric acid, heat shock proteins (HSP), HMBG1, and calreticulin [which stimulate DC in varied manners including via purinergic receptors (19), CD91 (20), TLR engagement (21, 22), RAGE (23), and TIM-3 (24)], are sufficient to promote glycolysis and DC maturation in the manner achieved with PAMP-mediated stimulation. Second, tumor-derived DC, or DC cultured with tumors, have been shown to be recalcitrant to TLR-mediated induction of CD40, CD86, and IL-12 (25), suggesting that the induction of glycolysis via this pathway in DC may be compromised. The mechanisms that regulate TLR function in mature DC after exposure to tumors have yet to be been elucidated, though inhibition of TLR signaling by MSR1 (see Section "MSR1 and DC Function" below) may contribute. Third, tumors are highly competitive for glucose; thus the substrate for glycolysis may be unavailable for DC and therefore the TME may not be permissive for the aspects of DC activation that are dependent upon glycolysis.

AKT AND mTOR IN DC FUNCTION

TLRs activate PI3K in a MyD88-dependent manner (26). Similar to cancer cells (27, 28) and effector T cells (15, 29, 30), PI3K/AKT pathway has been implicated to play a pivotal role in controlling metabolic transition to glycolysis in TLR-stimulated DC (**Figure 1B**) (18). AKT promotes glycolysis in DC in part by increasing the expression of Glut-1 and likely activates mTOR. In T cells, AKT signaling promotes glycolysis by inducing the expression of rate-limiting enzymes such as hexokinase and phosphofructokinase (31) and activates mTOR. AKT is not the only driver of metabolic alterations in TLR-stimulated DC, as it is dispensable for the programed down-regulation of palmitate consumption after TLR stimulation. (18). Confounding data exist, however, about the contribution of mTOR (which is normally a downstream target of AKT) to DC immunostimulatory capacity as inhibition of mTOR by rapamycin in murine GM-CSF-driven DC and human myeloid DC prolongs their lifespan, promotes expression of co-stimulatory molecules and cytokines, and enhances DC immunogenicity (32, 33). Mouse DC treated with rapamycin were more effective at generating tumor immunity compared to untreated controls. (32). However, in contrast, rapamycin-treated monocyte-derived human DC expressed significantly lower levels of pro-inflammatory cytokines and had reduced capacity to elicit $CD8^+$ T cell responses (33). Thus, while the TLR-induced activation of DC is dependent upon AKT-mediated induction of glycolysis, the contribution of mTORC1 [which is required to sustain glycolysis and effector functions in T cells (34)] to the glycolytic switch is unresolved, and sustained mTORC1 activation

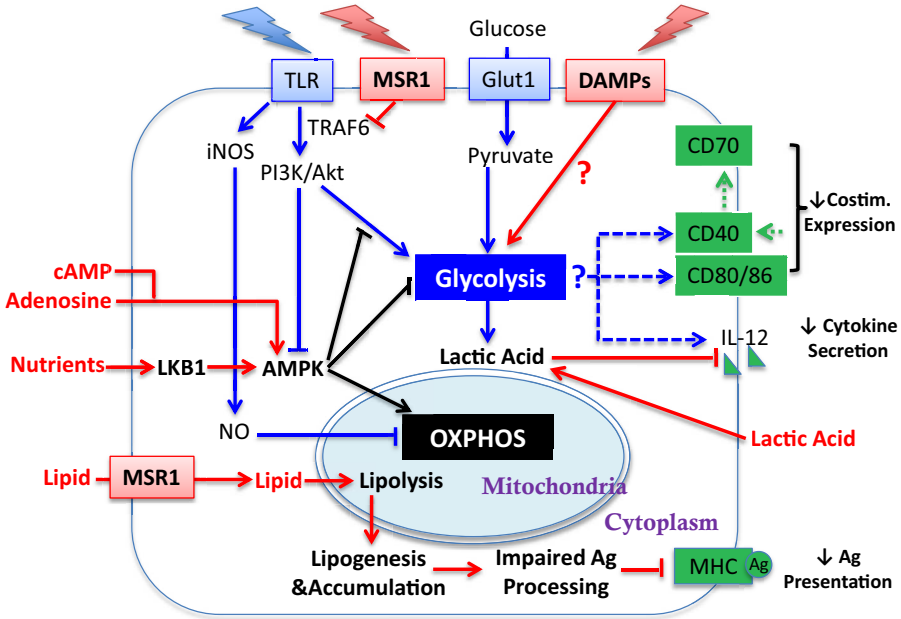

FIGURE 2 | Influence of tumor-derived factors on metabolic regulation of DC activation. Illustrated here are tumor-derived factors (in red) and their impact/potential impact on metabolic pathways (linked by red arrows) for DC activation. Other symbols are defined the same way as in **Figure 1**. Lipid accumulation is detrimental to DC activation by impairing Ag processing. How the TME influences glycolytic switch and how that impacts DC activation requires further investigation (see Sections "Requirement for Glycolysis and DC Activation," "Regulation of OXPHOS in DC," and "AMPK Regulation of DC Function").

appears detrimental to the function of DC. Further studies are also needed to dissect the contribution of mTORC2 to any of these processes.

REGULATION OF OXPHOS IN DC

The underlying mechanisms for the AKT-independent reduction in OXPHOS upon TLR stimulation of DC have been recently studied using real-time metabolic flux analysis (35). The progressive impairment in OXPHOS in TLR-stimulated DC is due to inducible nitric oxide synthase (iNOS)-derived NO (**Figure 1B**). LPS stimulation induces *NOS2* mRNA and iNOS protein expression, and subsequent NO production in DC, presumably via IFN-1 or NF-κB-dependent mechanisms as reported in macrophages (36). The autocrine NO causes mitochondrial impairment and blocks OXPHOS, as reported previously in astrocytes (37) and macrophages (38). The mechanism of OXPHOS inhibition is likely by NO reversibly competing with oxygen to inhibit cytochrome *c* oxidase, the terminal enzyme of the electron transport chain (39). Thus, the increase in glycolysis in DC after TLR stimulation could be a survival response that serves to maintain cellular ATP levels and to prevent cell death when OXPHOS is blocked and pyruvate accumulates. Most interestingly, although switch to glycolysis has been demonstrated to be a direct consequence of iNOS-mediated OXPHOS blockade and is essential for the survival of iNOS-expressing DC *in vivo*, a long-term switch to glycolysis was shown to be dispensable for full DC activation. When NO production is inhibited, glycolysis is abrogated and β-oxidation is maintained in TLR-stimulated DC. Despite this, these DC showed unimpaired if not enhanced activation, as assessed by surface expression of MHC and co-stimulatory molecules, production of inflammatory cytokines, and capacity of DC to stimulate T cell proliferative responses at 24 h after TLR stimulation. Therefore, optimal DC function may require only a transient switch to glycolysis; this notion is consistent with the fact that either stimulation of CD40 (see below) or limiting mTOR activity promotes DC function, and raises an alternative hypothesis to explain DC dysfunction in tumors: that rather than being unresponsive to DAMPs, chronic exposure to DAMPS from tumors leads to a state of glycolysis-induced exhaustion or elimination of DC (**Figure 2**).

AMPK REGULATION OF DC FUNCTION

The switch to glycolysis by DC is antagonized by adenosine monophosphate–activated protein kinase (AMPK), a master regulator of catabolic metabolism/OXPHOS in eukaryotic cells (**Figure 1**) (40, 41). AMPK can be induced by the nutrient sensor LKB1 (42), and functions in an opposing fashion with PI3K/AKT pathway to regulate TLR-induced metabolism and DC activation (**Figure 2**): up-regulation of AMPK in DC resulted in decreased LPS-induced IL-12p40 expression and glucose consumption, while suppression of AMPK by shRNAi leads to increased IL-12p40 and CD86 (18). Given the capacity of tumors to compete for glucose, one possible explanation for limited DC activity within the TME is the dominance of AMPK signaling over AKT-driven pathways (**Figure 2**). Intriguingly, IL-10, an inhibitor of DC activation, has been found to antagonize the TLR-induced hypophosphorylation of AMPK in TLR-stimulated DC, subsequently inhibiting the induction of glycolysis (18). AMPK activity is also strongly

induced by cAMP (**Figure 2**). Along with adenosine, cAMP can skew myeloid cell differentiation to a tolerogenic DC format (43, 44) and ablate the function of already differentiated DC (45). It is therefore intriguing to consider whether the negative regulatory activity of cAMP and adenosine in tumor immunity is mediated by abrogating glycolysis. However, the increased immunostimulatory capacity of DC after mTOR blockade or iNOS inhibition is not simply due to a restoration of β-oxidation. If this were the case, then contrary to these observations, AMPK activation should promote DC immunostimulatory capacity. Further, ligation of CD40, which signals via TRAF-6, has a major influence on DC activation state and viability (46–49), and is a potent promoter of co-stimulatory molecule and IL-12 expression and immunity to cancer. TRAF-6 mediated signaling has been shown to promote fatty acid oxidation in $CD8^+$ T cells (50) via activation of AMPK, raising the question as to how signals generated by CD40 engagement might be integrated into the metabolic programing initiated by TLR signaling. AMPK agonists have been proposed as anti-cancer agents due to their anti-Warburg effect in cancer cells (51), but this approach may be compromised by the detrimental effects of AMPK on DC and $CD8^+$ T cell function. The role of AMPK in regulating glycolysis and β-oxidation in early and late stages of DC function requires further elucidation before it can be predicted how such an approach would impact on DC function in tumors.

LACTIC ACID REGULATION OF DC FUNCTION

Additional metabolic regulation of DC has been described. Endogenously produced lactic acid, the end product of glycolysis, accumulates in dense monocyte-derived DC cultures and tumor spheroids (52). Lactic acid concentration after glycolysis, rather than oxygen availability, skews DC differentiation into a tolerogenic orientation, as exemplified by increased production of IL-10 and loss of IL-12 (**Figure 2**) in response to TLR stimuli (53). This potentially identifies a negative feedback loop in DC function induced by glycolysis within activated DC, and may suggest that the beneficial effect of preventing the switch to glycolysis achieved by inhibiting NO production *in vitro* (35) could be a secondary consequence of avoiding lactic acid accumulation in DC culture. Interestingly, Ag uptake, MHC class I presentation and co-stimulatory molecule (CD40 and CD86) expression on DC can be increased by acidosis/extracellular acid (54), via acid-sensing ion channels (ASICs) (55). Thus, acidity and lactate accumulation may be independent variables on DC maturation. Although lactic acid buildup due to excessive DC density is unlikely to be a major consideration *in vivo*, tumor-generated lactic acid may serve this purpose (**Figure 2**) (27, 56, 57). Lactate export by cells is passive, mediated by monocarboxylate transporters (MCTs). A high extracellular lactate concentration in the TME could prevent its export from glycolytic DC, leading to lactate accumulation. It is also worth considering whether lactic acid buildup will differentially affect DC that are resident within tumors compared to those at the tumor edge, and whether lymph node resident DC are vulnerable to lactic acid prior to lymph node invasion by metastatic disease. Further investigations are necessary to establish a comprehensive understanding about how changes in glycolysis and OXPHOS influence activation and/or survival of different TADC subsets, whether DC maturation states are equivalently influenced by metabolic alterations, and whether diverse TLR and other PAMP stimuli have similar impact on DC metabolism.

LIPID UPTAKE AND METABOLISM IN TADC

While a switch to glycolytic metabolism is generally consistent with immune cell activation, fatty acid metabolism, and lipogenesis are thought to promote quiescence (17, 50). Several studies have now begun to illuminate a rather complex role of lipid, and lipid accumulation, in DC function, and how the presence or production of triglycerides (triacylglycerol, TAG) in the context of tumors may influence DC function.

As DC develop and mature, particularly after LPS stimulation, they take on a "lacy" appearance that is composed of an increased presence of fat and glycogen-containing lipid-body droplets (58). Notably, these high lipid DC (HL-DC) express higher levels of scavenger receptors including MARCO/MSR1, which may contribute to their accumulation of lipid (58). Aside from serving as a building block for many facets of DC biology, lipid can contribute to critical aspects of the ability of DC to perform their Ag processing and presentation functions. Cross-presentation of exogenous Ag on MHC class I molecules is highly dependent upon the presence of lipid bodies. Genetic inactivation of genes that regulate lipid-body assembly, or the use of diacylglycerol acyltransferase inhibitors that prevent TAG accumulation, abrogates the MHC class I cross-presenting capability of DC (59). Thus, lipid production and consumption play critical roles in DC biology.

LIPID-MEDIATED INHIBITION OF TADC FUNCTION

It is therefore of interest that elevated levels of lipid, particularly TAG, were observed first by Herber and colleagues in DC during tumor progression of lymphoma, colon, and breast cancer in preclinical mouse models and cancer patients (60). The observed increased lipid accumulation is primarily a consequence of increased lipid uptake via up-regulated scavenger receptor A (SRA/MSR1/CD204) (**Figure 2**). Remarkably, considering the data from Bourgneres et al. (59), the major functional defect in HL-DC was a reduced capacity of DC to cross-present Ag (60). Normalization of lipid levels by a pharmacological inhibitor of acetyl-CoA carboxylase-1 (ACC-1), an enzyme that plays a critical role in lipogenesis, restored functional activity of lipid-laden DC, and enabled them to become more potent when used in a cancer vaccine (60). There are several notable aspects of this study that are worthy of further consideration. First, as mentioned above, lipid in DC by itself is not necessarily a marker of dysfunction. Indeed, a recent study examined the immunogenic qualities of liver-derived DC containing high and low amounts of lipid. HL-DC were considerably more immunogenic than their low lipid counterparts across multiple measurements (61). Further, Hwang and colleagues have demonstrated that saturated fatty acids can activate TLR4, leading to the up-regulation of MHC and costimulatory molecules. In contrast, polyunsaturated fats such as DHA, counteract the ability of saturated fats to induce DC maturation (62). Thus, rather than the amount of lipid within a DC being detrimental to function, the process by which lipid is acquired, or synthesized, or the type of lipid (saturated versus unsaturated) may be influential on DC function.

MSR1 AND DC FUNCTION

MSR1 has been shown to act both as a lipid receptor and as an innate pattern recognition receptor (PRR) that regulates inflammatory responses. As the first receptor identified for modified lipoproteins, the role of MSR1 has been well explored in pathogenesis of vascular disease particularly atherosclerosis (63). Besides modified self macromolecules, a wide range of PAMPs have been identified as MSR1 ligands, including bacterial surface components (e.g., LPS) and nucleic acids (e.g., CpG DNA and double-strand RNA), apoptotic cells, and endogenous danger molecules (64). Notably, the first report about negative effects of MSR1 in DC activation and function during adaptive immunity was presented by Yi and colleagues (65), in which they demonstrated that MSR1 suppresses the ability of TLR4 stimulation to license DC to prime naive CD8 T cells, drive their expansion, and promote their cytotoxic functionality both *in vitro* and *in vivo* (65). In agreement with data from Herber et al. they showed that lack of MSR1 in hematopoietic cells promoted tumor-protective immunity in a B16-OVA mouse melanoma model. In this model, MSR1 suppressed TLR4-induced activation of the transcription factor NF-κB by directly inhibiting ubiquitination of TRAF-6 (**Figure 2**) (66). However, the restriction of NF-κB activity by MSR1 can be independent of its ligand-binding domain, implying a novel signaling-regulatory role of MSR1 that can be uncoupled from its conventional role in endocytosis, including lipid uptake. Accordingly, one can speculate that up-regulation of MSR1 can contribute to DC dysfunction in cancer by skewing at least two pathways: (1) accumulation of lipids (2) suppression of TLR signaling. With respect to the second point, inhibition of TLR signaling may alter the balance between lipolysis and lipogenesis in favor of lipid accumulation. The therapeutic relevance of MSR1 and lipid uptake is reinforced by studies showing that direct targeting of MSR1 promotes tumor immunity (67, 68). Further, recent studies by Lerret et al. showed that the ability of total body irradiation (TBI), in combination with adoptive transfer of tumor-specific $CD8^+$ T cells, to control established breast tumors may be achieved by promoting activation and function in tumor-resident DC via down-regulating MSR1 and inhibition of lipid uptake (69, 70). However, the tumor-derived factors that up-regulate MSR1 are poorly characterized, and it is yet to be definitively shown that lipid is an immunoregulatory ligand for MSR1on DC.

MSR1-INDEPENDENT EFFECTS OF LIPID ON TADC

Although MSR1 engagement could account for poor DC function, additional influences of lipid on DC cannot be ruled out. Inhibition of ACC-1 resulted in normalization of lipid levels in TADC and was sufficient to restore functional activity in lipid-laden DC without changing expression of MHC and costimulatory molecules (60). This indicates that at least some accumulation of lipid in DC is due to *de novo* lipogenesis (**Figure 2**). Further, either the detrimental effects of lipid accumulation can be independent of MSR1 (as ACC-1 inhibition refunctionalizes TADC), or pathways released by ACC-1 inhibition can overcome MSR1-mediated inhibition. Evidence for the latter concept has been provide by Rehman et al. in a study demonstrating that ACC-1 inhibition enhances Ag capture (rather than Ag processing) by human DC (71). Confounding our understanding is that ACC-1 regulates the production of malonyl CoA, which in turn inhibits the activity of Carnitine palmitoyltransferase Ia (Cpt1a) (**Figure 1A**). Cpt1a strongly suppresses glycolysis via the Randle cycle, and knockdown of Cpt1a has been shown to strongly promote glycolysis in T cells (72). Thus, it is unclear why the inhibition of ACC-1, which should reduce glycolysis, enhances DC function unless (1) the lipogenesis program activates pathways that are significantly deleterious to DC function; (2) sustained glycolysis is indeed detrimental to DC function (discussed above); or (3) the presence of lipid is the detrimental factor, by influencing the availability of pyruvate for glycolysis rather than OXPHOS (73). Pointedly, it is uncertain why the accumulation of lipid might be detrimental to DC function at the level of Ag processing and presentation, especially given the importance of lipid bodies in this process. However, it has been shown that ceramides, which due to their hydrophobicity could accumulate in fat droplets, abrogate the ability of DC to uptake and present Ag (74) and also promote tumor-induced DC apoptosis (75).

IN VITRO VERITAS?

While the emerging picture of how alterations in DC metabolism can influence the function of DC, several words of caution should be written. One noticeable aspect of the majority of the studies cited in this review is that analysis of the contribution of metabolic alterations to DC function has generally been performed on DC generated from bone marrow or PBMC. This is necessitated by the rarity of DC in tissues, and the low sensitivity of the assays that are currently available to characterize metabolic activity. Thus, it is possible to posit that some metabolism-associated alterations described in these studies could be dependent upon the culture conditions that generate or sustain DC, and extrapolation to *in vivo* DC, particular to intratumoral DC, is not yet merited. DC, particular those of murine origin, generated via culture exist in a semi-activated functional state (our unpublished data) that may lead to different metabolic choices, and be influenced by different stimuli, compared to truly immature DC. This point is particularly salient when we consider some of the reported discrepancies on the impact of limiting or promoting glycolysis by modulating mTOR activity. Further, much work has yet to be done in defining whether metabolic alterations actually promote discrete functions of DC, or whether metabolic switching is a response to alterations in the nutrients in the immediate environment of the DC (tissue; lymphatics; lymph nodes, for example). However, the capacity for TLR to induce metabolic changes in DC in the consistent nutrient environment provided by *in vitro* culture, suggests that metabolic changes are not entirely due to alterations in the available nutrients, but rather these metabolic changes directly impact/regulate the activation and survival of DC. The single-cell analytical luxuries provided by flow cytometry have yet to be translated to metabolism studies, limiting our ability to make direct assessment of *in vivo* DC metabolic changes. Unfortunately, until radiotracer incorporation, extracellular flux assays, and mass spectrometry can be applied to 1000s of cells, rather than 100,000s, we will be dependent upon the use of fluorochrome-labeled substrates such as the glucose-derivative 2-NBDG to guide our impression of the metabolic pathways being used by DC derived from different *in vivo* environments.

SUMMARY

The metabolic and biochemical regulation of DC activation, function, and survival are just the beginning to be elucidated. Further understanding of this process will likely improve the quality and efficacy of DC expanded *ex vivo* for cancer vaccines [note the varied influences of cytokines on vaccine efficacy (9, 76)], as cytokines are known to impact metabolism. Further, metabolic re-invigoration of DC may provide an avenue for enhancing DC function in the TME or in tumor-draining lymph nodes, allowing for increased Ag processing and presentation after the induction of tumor damage, or in association with inhibition of checkpoint blockade molecules. Finally, approaches that promote the availability of glucose, or limit lipid uptake, in the TME might well increase the ability of TADC to activate and contribute to the adaptive immune responses elicited against tumors.

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Immature, semi-mature, and fully mature dendritic cells: toward a DC-cancer cells interface that augments anticancer immunity

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Dendritic cells (DCs) are the sentinel antigen-presenting cells of the immune system; such that their productive interface with the dying cancer cells is crucial for proper communication of the "non-self" status of cancer cells to the adaptive immune system. Efficiency and the ultimate success of such a communication hinges upon the maturation status of the DCs, attained following their interaction with cancer cells. Immature DCs facilitate tolerance toward cancer cells (observed for many apoptotic inducers) while fully mature DCs can strongly promote anticancer immunity if they secrete the correct combinations of cytokines [observed when DCs interact with cancer cells undergoing immunogenic cell death (ICD)]. However, an intermediate population of DC maturation, called semi-mature DCs exists, which can potentiate either tolerogenicity or pro-tumorigenic responses (as happens in the case of certain chemotherapeutics and agents exerting ambivalent immune reactions). Specific combinations of DC phenotypic markers, DC-derived cytokines/chemokines, dying cancer cell-derived danger signals, and other less characterized entities (e.g., exosomes) can define the nature and evolution of the DC maturation state. In the present review, we discuss these different maturation states of DCs, how they might be attained and which anticancer agents or cell death modalities (e.g., tolerogenic cell death vs. ICD) may regulate these states.

Keywords: immunogenic cell death, phenotypic DC maturation, cytokine, antigen, cell death, cancer, immunosurveillance, chemotherapy

INTRODUCTION

It is conceptually established that the immune system can be distributed across two basic components, i.e., the innate immune system and the adaptive immune system (1, 2). The primary aim of innate immune cells is to provide a rapid non-specific response to any pathogen or foreign aggressors (possessing foreign antigens), wound, inflammatory insult, or newly initiated diseased cell (owning possible "non-self" antigens) (1, 2). On the other hand, the primary aim of adaptive immune cells is to provide a latent but highly specific response against foreign or "non-self" antigens and to generate an "immune memory" against these antigens to counter similar insults in the future more quickly (either cell interaction dependent or independent; the latter applying to antibody production) (3, 4). Together these two branches of the immune system are supposed to initiate acute inflammation ultimately culminating in its resolution and wound healing once they have taken care of the aggressor, insult, or diseased cell (5, 6). It is noteworthy that in terms of evolution, the conception of the innate immune system pre-dates that of the adaptive immune system (1). Most notable innate immune cells include macrophages, natural killer (NK) cells, dendritic cells (DCs), various myeloid lineage subsets, neutrophils, basophils, and eosinophils (1, 6); while the most notable adaptive immune cells include T and B lymphocytes (3, 5).

The initial reaction orchestrated by innate immune cells consists of capturing, as well as clearing up or destroying the source of injury, infection, or diseased cells, followed by wound healing and if required (in case of well discernable antigens) "priming" of the adaptive immune cells against antigens derived from the "non-self" diseased cells or pathogens (1, 2). This adaptive immune cell priming helps to initiate more specific responses, directed against the acquired antigens and leading to the eradication of the antigen source (3, 6). This in principle is also the basic theory behind anticancer immunity or anticancer immunosurveillance (7), where innate immune cells recognize the "non-self" tumor-associated antigens (TAAs) and prime adaptive immune cells (mainly T cells) against them. This leads to both: direct and indirect cancer killing,

Abbreviations: APC(s), antigen-presenting cell(s); CD, cluster of differentiation; DAMPs, damage-associated molecular patterns; DC(s), dendritic cell(s); DEX(s), dendritic cell-derived exosomes; ER, endoplasmic reticulum; GM-CSF, granulocyte macrophage colony stimulating factor; Hyp-PDT, hypericin-based photodynamic therapy; ICD, immunogenic cell death; iDC(s), immature dendritic cell(s); IFN, interferon; IKDCs, IFN-producing dendritic cells; IL, interleukin; imDEXs, immature dendritic cells derived exosome(s); LFA-1, leukocyte function-associated antigen-1; mDEXs, mature dendritic cells derived exosome(s); MDSC, myeloid derived suppressor cells; NK, natural killer cells; NKDCs, natural killer dendritic cells; NLRs, NOD-like receptors; PAMPs, pathogen-associated molecular pattern(s); PRRs, pattern recognition receptors; TAA(s), tumor-associated antigen(s); TDE(s), tumor derived exosome(s); TGF, transforming growth factor; TIDCs, tumor-infiltrating dendritic cells; TLRs, toll-like receptors; TNF, tumor necrosis factor.

anticancer effector functions, production of anti-TAA antibodies and subsequent immunity capable of rejecting tumor cells possessing the corresponding TAAs (3, 8). In this complex interplay, one may appreciate that the step of "priming" adaptive immune cells by innate immune cells against TAAs represents a crucial milestone that is completely dependent on the antigen-presenting and antigen-sensing capabilities of innate immune cells (2). While most innate immune cells (professional presenters) and certain cells of epithelial lineage (non-professional presenters) are capable of presenting antigens to the adaptive immune cells (6) be it to varying degrees; yet the sentinel antigen-presenting cells (APCs) of the immune system are the DCs (2, 3, 9). DCs are the guardian APCs because they are both efficient at antigen-presenting and adaptive immune cell activation and also good at judging whether an entity possesses "self" or "non-self" antigens (2, 10, 11). The ability of DCs to present "non-self" TAAs properly to prime as well as to activate adaptive immune cells is an absolute pre-requisite for activation of potent anticancer immunity (2, 4).

In the present review we briefly discuss the basic biology of DC activation states that can make a difference between pro-tumorigenic inflammation and anti-tumorigenic immunity. We will then discuss in more detail the ability of anticancer therapeutics to influence and modulate these activation states and the crucial impact of exosomal communication on DC-associated functions.

DENDRITIC CELLS AND THEIR ACTIVATION STATES: A BIRD'S EYE-VIEW

The molecular cell biology of DCs has evolved in a sophisticated manner to facilitate its APC functions (12). DCs in general possess a diverse repertoire of surface receptors (and intracellular receptors) that help them in environmental sensing and to carry out "at will" rapid innate immunity-related functions (2, 12). Such receptors include various scavenging or phagocytic receptors like CD91, integrins, CD36 (aiding in phagocytosis and clearance of target entities), surface pattern recognition receptors (PRRs) like toll-like receptors (TLRs), and intracellular PRRs like NOD-like receptors (NLRs) (10, 13, 14). DC-based PRRs help in detection (and subsequent DC stimulation) of danger signals like pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (4, 5, 8).

Dendritic cells are also special in terms of their antigen processing machinery. Classically (for non-professional APCs and normal cells, as applicable), antigens derived from intracellular sources are presented by the major histocompatibility complex (MHC) class I presentation system while extracellular antigens (captured *via* phagocytosis or pinocytosis) are preferentially processed for MHC class II presentation (15). In specialized APCs like DCs however, the extracellular antigens can also gain access to the MHC class I presentation system (mediated by following events: phagophore → endosome → antigen escape from endosome → antigen processing by cytosolic proteasome for MHC I presentation) while intracellular antigen fragments can also be found on the MHC class II molecules (mediated by autophagy) – a phenomenon termed as "cross-presentation" (15). This unique ability to cross-present antigens to adaptive immune cells is also behind DCs' significant role as APCs. Depending on the environment they encounter (e.g., normal "self" antigen rich environment or abnormal "non-self" antigen rich environment); DCs can exhibit various states and accordingly perform different functions (2, 12). Based on a highly stark difference between antigenic environments, i.e., host "self" antigens vs. foreign or pathogen-associated "non-self" antigens, DCs can exist in two main states, i.e., steady state immature dendritic cells (iDCs) and fully mature DCs (9, 12). The distinction between immature and mature DCs is partly based on changes occurring on two crucial levels, i.e., phenotypic level and functional level (2, 14, 16). Phenotypic maturation is attained when DCs up-regulate surface maturation ligands such as CD80, CD83, and CD86 along with the MHC class II molecule (9). DCs stimulated on the functional level exhibit the ability to secrete cytokines where the balance between inflammatory or immunostimulatory cytokines (e.g., IL-12, IL-6, IL-1β) and immunosuppressive cytokines (e.g., IL-10, TGF-β) is decided by the "environmental context" (2, 9, 17).

In normal, healthy conditions, DCs exist in an immature or steady state such that in this scenario their main aim is to maintain immune tolerance by impeding adaptive immune cells from attacking host cells that possess "self" antigens (4, 10, 12). However, if DCs encounter "non-self" entities in the periphery, they opsonize them, process their antigens for cross-presentation, migrate to the lymph nodes, and prime naïve T cells for these antigen (9). DCs provide the T cells with the information about whether an antigen is present and whether it poses a threat – a foundational mechanism for the subsequent T cell effector function (18). A single DC can contact as many as ∼5000 T cells per hour (19). Steady state iDCs exhibit continuous endocytic activity (20) and hence continuously present "self" antigens to T cells. However in this case the T cells are not polarized toward an effector state but are rather polarized to facilitate tolerance or immunosuppression (12, 21). Such immunotolerance is actively induced and maintained through a mixture of immune checkpoint pathways and complete lack of stimulatory signals provided by the DCs (22). Immune checkpoint pathways are a plethora of inhibitory cascades that are crucial for maintaining self-tolerance and modulation of duration/amplitude of immune response, e.g., DC-based presentation of ligands like cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programed cell death protein 1 (PD1) to T cells causing T cell anergy or differentiation of immunosuppressive T cells (22). Such immunosuppressive T cells (e.g., regulatory T cells, T_{regs}) further help in spreading tolerance toward "self-antigens" (6, 9). On the other hand, when DCs encounter pathogens or entities possessing PAMPs (detected in part through PRRs) they switch to a mature state exhibiting strong phenotypic and functional stimulation. At this stage, the DCs leave the function of phagocytic scavenging and assume the more sophisticated APC-function (12). Subsequently, DCs carefully co-ordinate their proteolytic processes in the cytosol (e.g., proteasomes), endosomes-lysosomes (e.g., lysosomal hydrolases), and the endoplasmic reticulum (ER) to degrade "non-self" entity-derived proteins in order to yield suitable antigenic peptides that are subsequently loaded on MHC class I and II molecules for presentation to T and B cells (9, 12). The simultaneous presence of phenotypic maturation ligands, suitable cytokines, other functional immunostimulatory factors, and appropriate antigen-MHC

complexes helps activate an effector profile in interacting T cells thereby polarizing them for antigen-specific elimination of the "non-self" entity (9). Here, antigen-MHC complexes are the main stimulatory signals (signal 1, detected by the T cells through a complex of T-cell receptors/TCRs-CD3) followed by phenotypic maturation ligands. These ligands help in providing proper co-stimulation by binding corresponding receptors on T cells (signal 2, detected by T cell receptors like CD28, CD40L) in the presence of cytokines or factors eliciting immunostimulation and the effector T cell phenotype (signal 3, detected by respective cytokine cognate receptors) (9). The presence of these three signals is absolutely essential for effective T cell stimulation by APCs (like DCs) and their polarization toward anti-pathogenic effector function (6, 9). It is noteworthy though, that apart from these three signals, DCs might modulate T cell function via other immunomodulatory signals (e.g., exosomes, discussed later); however because they still lack a well-characterized functional status, they cannot yet be ascribed as *bona fide* T cell modulatory signals. Last but not least, it is important to consider that maturation of DCs is primarily crucial for the activation and differentiation of naïve T cells (10). Pre-existing cytotoxic T cells and memory T cell populations are not very strongly dependent on fully mature DCs for their effector functions (2, 3, 12).

TUMOR-INFILTRATING DCs: AN OVERVIEW

The Dichotomy of DC maturation states is mainly applicable to an environment where a very obvious distinction exists between "self" and "non-self" antigens. The continuum of DC activation states is much more complex when it comes to cancer as most cancerous tissues or tumors are very similar in terms of antigenic make-up to that of normal cells (5, 12). This is attributable to the fact that most antigens are either shared with nearby normal tissues (e.g., differentiation antigens) or with spatiotemporally distinct yet normal tissue [e.g., oncofetal antigens or cancer-testis antigens (7)]. This leads to a strong conflict regarding what represents "self" or "non-self" – which is further revived by the struggle between the tolerance-encouraging tendency of DCs and their propensity to prime T cells for tumor rejection (4, 9, 12). This situation is further exacerbated by the capacity of cancer cells to interfere with normal DC function (23) *via* immunosuppressive cytokines or other signals like those conveyed by exosomes (discussed later).

In a well-established tumor, cancer cells actively suppress steady state DCs (also called tumor-infiltrating DCs or tumor-infiltrating dendritic cell, TIDCs) and keep them in the favorable immature state (**Figure 1**) (23–25). Such immature TIDCs tend to exhibit dysfunction in antigen-presenting capabilities, suppressed endocytic activity, abnormal motility, and various other immature characteristics – a point that has been demonstrated in a number of studies analyzing various solid tumors and tumor-draining lymph nodes (26). Such induction of immature state in TIDCs by the tumor is not surprising considering that mature DC's density in tumors inversely correlates with tumor pathologic grade/stage and positively correlates with improved prognosis (26). Moreover, tumors may also actively induce apoptosis in TIDCs through certain gangliosides (e.g., GM3, GD3), glycoproteins (e.g., MUC2 mucins), and neuropeptides (25, 26).

The tumor-induced iDCs state is mainly characterized by: (1) the total absence or presence of negligible amounts of well-processed cancer antigens (compromised signal 1 generation), (2) absence or trivial amounts of phenotypic maturation ligands or co-stimulatory molecules (ablation of signal 2), and (3) either complete absence or minor presence of functional stimulus/immunostimulatory cytokines like IL-12 (ablated signal 3) (7, 10, 12, 23). Such iDCs can also be encouraged by the presence of non-immunogenic cancer cell death [e.g., tolerogenic apoptosis (11)] (5, 21, 27, 28). The presence of signal 1, i.e., processed cancer antigens is very crucial for potent elicitation of anti-tumor immunity since signals 2/3 have less meaning in absence of signal 1 (18). Thus not surprisingly, one of the immunoevasive strategies employed by cancer cells is the down-regulation or loss of antigens (7, 21). DCs prime the T cells for cancer antigens in the lymph nodes in three phases (18, 29); Phase I lasts for ∼8 h and consists of transient interactions between T cells and antigen-presenting DCs (29). T cells integrate antigenic stimulus from several such Phase I encounters until the cumulative signal triggers the onset of Phase II. During Phase II (which lasts ∼12 h), T cells form a long-lasting stable contact with a single DC (29). It is noteworthy that this Phase I–II transition depends strongly on the concentration of antigenic peptide-MHC complex per DC (18, 30); higher the concentration, the faster the tendency of T cells to exit Phase I and reach Phase II (18). Thus, lower cancer cell-associated antigen levels make it harder for the T cells to exit Phase I – a scenario that leads to unstable DC–T cell interactions and compromised T cell immunity. Phase II is also the stage where T cells are further activated *via* DC-based signals 2 and 3 (29). Thereafter, the T cells enter Phase III during which they proliferate vigorously and return to short interactions with the DCs (29). It should be note however, that the above "three phase theory" of DC–T cell interactions is mainly based on *in vitro*/*ex vivo* studies using either model antigens or high concentrations of TAA-based immunodominant peptides. Such studies need to be extended to settings of DC–T cell interactions within a tumor-bearing host, in near future.

Apart from antigen down-regulation, cancer cells also directly induce an immature TIDC state through secretion of immunosuppressive factors like IL-10, VEGF, TGF-β, and PGE_2 (7, 25, 27); thereby further compromising stable DC–T cell interactions. The strategies and mechanisms employed by cancer cells for inducing DC-based tolerogenicity have been discussed in details in certain recent reviews (5–7, 21). Curiously it has been demonstrated recurrently that in an *ex vivo* set-up, certain iDCs may exhibit the ability to directly lyse transformed cells or tumor cells *in vitro* (31). Such iDCs have been termed as natural killer dendritic cells (NKDCs) or more recently interferon-producing killer DCs (IKDCs) (**Figure 1**) (31) and have been found to exert anticancer cytotoxic activity *in vitro* in both rodent and human set-ups (31–33). While, IKDCs may simply reflect the prevalent *ex vivo* DC heterogeneity yet their characterization raises the need to better study DC features in tumor-bearing hosts.

DC ACTIVATION STATES IN TUMOR IMMUNOSURVEILLANCE AND ANTI-TUMOR IMMUNITY

As per the theory of cancer immunoediting, during tumor development the equilibrium between growing tumor and immune

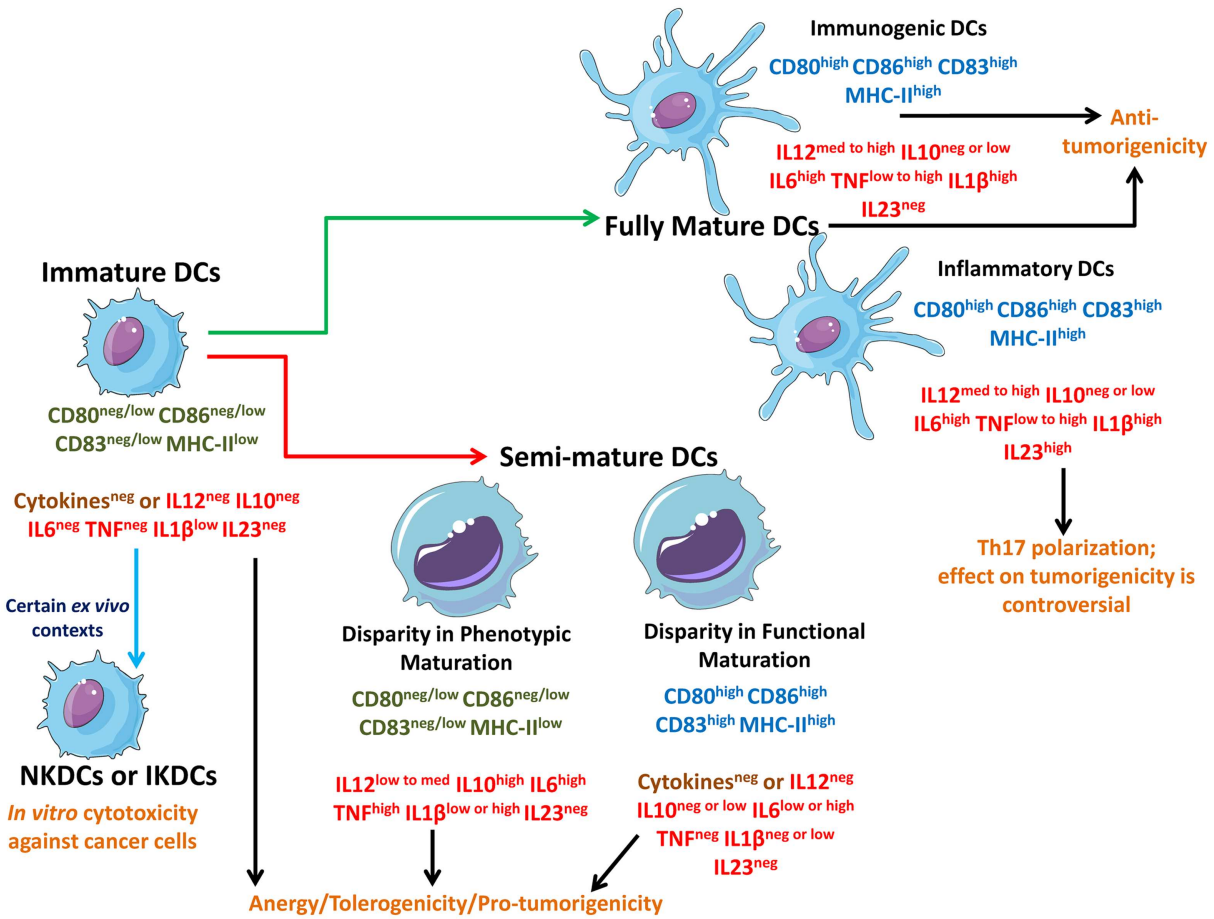

FIGURE 1 | Schematic representation of different states of DCs interacting with different types of cancer cells. Live cancer cells and cancer cells undergoing non-immunogenic cell death keep the steady state DCs in an immature state devoid of strong up-regulation of phenotypic maturation ligands (CD80, CD86, CD83, MHC-II) and functional maturation (no or negligible amounts of immunostimulatory cytokines). In certain *ex vivo* conditions, immature DCs can behave like natural killer DCs (NKDCs) or interferon-producing killer DCs (IKDCs), which can exert *in vitro* anticancer cytotoxicity. On the other hand, freeze/thawing of cancer cells, certain immunogenic live cancer cells, and certain therapy-induced non-immunogenic cell death routines can induce a "limbo" state in DCs called semi-mature DCs which are not fully mature and can be either devoid of phenotypic maturation ligands or functional maturation depending on the context. Both immature DCs and semi-mature DCs cause T cell anergy and facilitate tolerogenicity thereby compromising anticancer immunity. These DCs may also actively facilitate pro-tumorigenic signaling. However, some therapeutic paradigms can induce immunogenic cell death (ICD) or at least a certain level of augmented immunogenicity in cancer cells which causes the interacting DCs to fully mature. These fully mature DCs can potently elicit anticancer immunity. Fully mature DCs devoid of immunosuppressive cytokines like IL-10 can be termed as immunogenic DCs capable of forming the most productive interface with T cells to prime them for anticancer effector function. On the other hand, fully mature DCs secreting IL-23 (inflammatory DCs) may polarize the T cells toward a state where they have a "helper" behavior accompanied by IL-17 production (Th17). The role of Th17 cells in cancer immunity and progression is enigmatic and controversial.

system shifts: at the beginning the immune system is capable of recognizing and exterminating cancer cells ("elimination" phase). Later, cancer "immunoediting" and release of cancer-derived immunosuppressive factors, results in the establishment of an equilibrium between cancer cells that are still susceptible to immunoeradication and immunoevasive ones that are resistant to anticancer immunity ("equilibrium" phase). Finally, as the immune evasion process progresses, the tumor escapes immune cell control ("escape" phase) (34). It has been long proposed that anticancer therapies should kill the cancer cells in a manner that helps activate the DCs to prime the adaptive immune system for anticancer activity (28, 35), however the experimental as well as clinical translation of this idea have unfortunately not been straightforward. This may result from the fact that most anticancer therapies tend to induce either non-immunogenic or very low-immunogenic cancer cell death (11) and thereby disallowing sufficient DC stimulation (5, 21, 27, 35) and keeps the DCs in an immature state (**Figure 1**). For instance, certain therapeutic modalities (e.g., chemotherapeutics like cisplatin) or certain anti-tumor vaccine-preparation methodologies (i.e., freeze/thawing, discussed later in Anticancer Therapy Differently Shapes the DC-Dying Cancer Cells Interface), may actually cause a sub-optimal activation of DCs (24, 28, 36, 37) thereby giving rise to a somewhat "limbo" state which can be termed as "semi-mature"

Table 1 | Inducers of cancer cell death that stimulates full maturation of DCs.

Anticancer therapy	*In vitro* Phagocytosis	Phenotypic maturation of DCs	Release of cytokines by DCs	Stimulation of T cells	*In vivo* mice experiments	Clinical data
Hypericin-PDT	Garg et al. (37)	Garg et al. (37)	IL-1β (37); IL-6 (50); NO (37); IL-12p70 (Dudek et al., unpublished data)	Proliferation (50) IFNγ release (50)	*In vitro*-treated cancer cells induce antitumor immunity in mice vaccination experiment (37)	
UVB	Kotera et al. (52)		IL-12 (52)		Pulsed-DC induce antitumor immunity in mice vaccination experiment (52)	
Cyclophosphamide (MAFO for *in vitro* experiments)		Kotera et al. (52), Schiavoni et al. (53)	IL-1β, IL-6, IL-12 (52, 53)			Increased infiltration of phenotypically mature DCs (53, 55–57); increased DCs trafficking to the lymph node (53)
γ Irradiation	Prasad et al. (58), Kim et al. (59)	Prasad et al. (58), Kim et al. (59)	IL-6 (59)	IFNγ release (62)	After tumor irradiation: (1) increase in tumor-infiltrating mature DCs (60, 61); (2) increase in IFNγ production by spleen cells (63)	
Doxorubicin	Obeid et al. (132)	Ghiringhelli et al. (134)	IL-1β (134)	Proliferation and IFNγ release (133)		
Oxaliplatin		Ghiringhelli et al. (134)	IL-12p70 (134)	Proliferation and IFNγ release (134)		
Bortezomib		Cirone et al. (135)		IFNγ release (135)	Pulsed-DC induce antitumor in mice vaccination experiment (136)	
CMQ and colchicines		Wen et al. (137)		Proliferation (137)		
Oncolytic viruses		Moehler et al. (73), Donnelly et al. (138)		Release of IFNγ (138), release of TNF and IL-6 (73)		

DCs (**Figure 1**) (10). It is noteworthy though that in certain instances, semi-mature DCs generated *ex vivo* and injected back into the host (in this case rhesus macaque) might become mature spontaneously during migration before reaching the lymph nodes (38). However, whether this situation applies to therapeutic DC vaccines is an enigmatic question since the above mentioned study was not done within the context of anticancer DC vaccines. In various anticancer therapy settings (see **Table 1** and Anticancer Therapy Differently Shapes the DC-Dying Cancer Cells Interface), DCs interacting with dead/dying cancer cells (treated with non-immunogenic or low-immunogenic anticancer agents) may attain a semi-mature state, i.e., while they may present low/medium levels of cancer antigens yet they either lack co-stimulatory signals (e.g., CD86) or suitable immunostimulatory cytokines (e.g., IL-12) (6, 10, 28, 37). Thus, semi-mature DCs, unlike iDCs, exhibit the ability to sustain at least two (i.e., signal 1 and either one of the other two signals) of the three signals required for successful/optimal T cell activation (23) but unfortunately not all three at once and thereby they exhibit an unstable interface with T cells that leads to active ablation of anticancer immunity (10) and clonal T cell anergy (20, 23, 24). Semi-mature DCs might exhibit inconsistency in either up-regulation of phenotypic maturation ligands or in secretion of cytokines (**Figure 1**). Semi-mature DCs with disparity in phenotypic maturation are able to secrete

one or more of the few assorted cytokines like IL-10, IL-6, IL-1β, and tumor necrosis factor (TNF), but do so to a highly variable degree (in terms of amount and simultaneous presence of these cytokines together) (23, 28, 37). It is also noteworthy that certain well-established tumors composed of immunogenic cancer cells (e.g., melanoma) may also encourage formation of *de novo* semi-mature TIDCs rather than immature TIDCs due to the particular tumor microenvironment they can create (39). Together iDCs and semi-mature DCs tend to encourage T cell anergy or T cell exhaustion (9, 10), tolerogenicity toward the cancer cell (9, 31), and even active pro-tumorigenic activity (e.g., semi-mature DC-derived IL-6 may act as a growth factor for tumors expressing IL-6R-gp130 cognate receptors and/or IL-10 can act as a general immunosuppressor) (17, 40, 41).

Recently however, it was described that certain therapeutic modalities [e.g., mitoxantrone/doxorubicin, hypericin-based photodynamic therapy (Hyp-PDT), and radiotherapy] cause cancer cells to undergo immunogenic cell death (ICD) (28, 35, 40, 42). ICD tends to be highly immunostimulatory because it emits a spatiotemporally defined combination of potent DAMPs that act as danger signals important for DCs stimulation (35). DCs detect such danger signals through a combination of receptors including TLRs, CD91, and purinergic receptors (21, 35). ICD may also ablate the canonical strategies harnessed by cancer cells to encourage the formation of immature or semi-mature DC states (21, 27). Beyond ICD, some anticancer therapeutics (e.g., antimitotic chemotherapeutics like docetaxel) may induce a general augmentation of immunogenicity that is not as strong as ICD but is still effective in a context-dependent fashion (43). Cancer cells undergoing ICD, or exhibiting therapy-induced (minor to medium increase of) immunogenicity, encourage the formation of fully mature DCs (**Figure 1**) (10, 27, 28, 35, 37, 43). In general, fully mature DCs exhibit all three conventional T cell stimulatory signals, thereby enabling elicitation of potent anticancer immunity (12, 13, 31). However, based on the pattern of only a few cytokines fully mature DCs might be subdivided, i.e., immunogenic DCs and inflammatory DCs (**Figure 1**) (35, 44). The fully mature immunogenic DCs are supposed to exhibit the least or total absence of immunosuppressive cytokines like IL-10 (17, 21, 40). Most known ICD inducers result in the formation of general fully mature DCs, with a context-dependent absence or reduced abundance of immunosuppressive cytokines (e.g., IL-10) (28, 37, 45). On the other hand, the presence of high IL-23 cytokine expression might be a marker of inflammatory DCs (44). Indeed, IL-23 may encourage T cells to exhibit the Th17 polarization (T helper cells/Th cells producing IL-17 cytokine) (44). It is noteworthy that the role of inflammatory DC-Th17 arc in cancer progression is still enigmatic with evidence supporting both anti-tumorigenic and pro-tumorigenic roles for this interaction, depending on the context (44, 46, 47). Thus for anticancer immunity, the functional role of fully mature inflammatory DCs needs to be treated with caution until further research ascertains their exact behavior.

It is noteworthy though, that the distinctions between different DC maturation or activation states made on the basis of phenotypic maturation markers or cytokine patterns are primarily based on *ex vivo* or *in vitro* experiments. This is because simultaneous analysis of various surface-associated and soluble DC activation markers is relatively easy *ex vivo* or *in vitro*. However, *in vivo* or *in situ*, such a simultaneous detection is nearly impossible. *In vivo* or *in situ*, mostly only the phenotypic maturation status of tumor-infiltrating DCs is detected *via* immunofluorescence staining (e.g., $CD11b^{+}CD11c^{+}CD86^{high}MHC\text{-}II^{high}$ DCs). While an analysis of cytokines associated with the tumor is possible via RT-PCR, proteomics-approaches, or antibody arrays, yet there is no way of characterizing which cytokines are secreted exclusively by the TIDCs. In future, lineage-tracing of the DCs in tumors or high enumeration staining/detection strategies for TIDCs might make it possible to simultaneously detect the phenotypic and functional markers of DCs *in vivo* or *in situ* however until that point, the above mentioned distinctions can be treated as operational definitions. Furthermore, it would be necessary to further characterize the additional states of semi-mature or fully mature DCs relevant for cancer treatment, not only *in vitro*/*ex vivo* but also *in vivo*/*in situ*.

ANTICANCER THERAPY DIFFERENTLY SHAPES THE DC-DYING CANCER CELLS INTERFACE

Anticancer therapies are capable of modulating DC states, either directly or *via* dying cancer cells. We believe that efficient anticancer treatment should be able to re-establish the recognition of cancer cells by the immune system, as well as "revive" the dominance of the immune system in this cross-talk. Therefore, the maturation status of DCs, as the predominant APCs, after anticancer therapy or after co-incubation with *in vitro*-treated dying cancer cells is an attractive marker of stimulation of an immune response, specifically relying on effector $CD4^{+}/CD8^{+}$ T cells (characterized by increased T cell proliferation/infiltration and secretion of IFNγ) (48).

Interestingly, cancer cells treated with most anticancer therapies either induce full DC maturation (a very small fraction of therapies) or do not stimulate the DCs at all (i.e., immature or tolerogenic DC formation, induced by a large fraction of therapies). There are however, a limited number of therapies that can also induce the formation of semi-mature DCs. In the next section, the formation of fully mature and semi-mature DCs will be discussed within the context of anticancer therapies.

FULLY MATURE DCs

Only few therapies have been reported to have the capability to induce cancer cell death that stimulates complete DC maturation. By complete maturation of DCs we understand induction of both, phenotypic markers and production of immune-stimulating cytokines. Instead, to the best of our knowledge, in most *in vitro* studies, the analysis of cytokine expression profile is either incomplete, or the most important cytokines, e.g., IL-12p70, IL-10, are not included. Only such fully mature DCs are able to stimulate T cells, hereby increasing their proliferation and secretion of IFNγ, which are often considered to be surrogate indicators of a productive immune stimulation. Thereby, in the absence of information on the full pattern of cytokines released by DCs', an increase in T cell stimulation can be considered a strong indicator of a full maturation state of the aforementioned DCs. Moreover, full maturation of DCs can be assumed with high probability if

anticancer immunity in syngenic mice vaccination models (e.g., B16 cells in C57Bl/6 mice, MCA205 cells in C57Bl/6 mice, CT26 cells in BALB/c mice, 67NR cells in BALB/c mice) is achieved when dying cancer cells, following chemotherapy *in vitro*, are administered (either in a prophylactic or curative set-up). An anticancer treatment that can induce productive maturation of dying cancer cell-loaded DCs, at least *in vitro*, is Hyp-PDT (37, 49–51). Already for some time it is known that Hyp-PDT-treated cancer cells induce both phenotypic and functional maturation of DCs (37, 50) and that in mice vaccination experiments, the dying cancer cells stimulate anticancer immunity preventing growth of transplantable tumors (37). Recently this data was re-confirmed and extended further (50). DCs interacting with Hyp-PDT-treated cancer cells exhibit a fully mature immunogenic phenotype functionally characterized by significant secretion of immunostimulatory factors like IL-1β, IL-6, nitric oxide, and the absence of the immunosuppressive cytokine, IL-10 (50). Moreover, Hyp-PDT-treated cancer cells elicit secretion of IL-12p70 by loaded DCs (Dudek et al., unpublished data).

Other treatments for which the detailed immune-effects have been described include UVB irradiation, cyclophosphamide, and γ-irradiation. There is evidence that UVB-induced dying cancer cells are phagocytosed by DCs, leading to an increase in IL-12 production (52). Furthermore, DCs pulsed with UVB-treated B16F10 cells, induce anti-tumor immunity in mice and prevent growth of transplantable tumors (52). As cyclophosphamide requires hepatic activation, for *in vitro* investigations its analog, MAFO, is used. Exposure of DCs to mafosfamide (MAFO)-treated cancer cells causes phenotypic maturation of DCs and their functional stimulation, characterized by the release of various cytokines (IL-1β, IL-6, IL-12) (53, 54). Moreover, the treatment with cyclophosphamide of tumor-bearing mice results in increased tumor bed infiltration by phenotypically mature DCs (53, 55–57), as well as increased trafficking of DCs from the tumor bed to the draining lymph nodes (53). Furthermore, cyclophosphamide, when given to patients at metronomic doses, combines direct effects on immune cells, like: limitation of T_{reg} cells population and expansion of DCs in peripheral blood (56, 57) with potent stimulation of a DC response. Also γ-irradiated murine melanoma cells are efficiently phagocytosed by DCs, resulting in their phenotypic maturation (58, 59). Despite the fact that neither IL-12p70 nor TNF are secreted by loaded DCs, these cells release another pro-inflammatory cytokine, IL-6 (59). These observations prove the triggering of a functional, however not optimal (lack of IL-12p70), maturation of loaded DCs. The positive immunostimulatory effects of γ-irradiation were shown by increased tumor-infiltrating active DCs following local high-dose radiotherapy (60, 61). Furthermore, when human monocyte-derived DCs and irradiated melanoma cells were co-incubated with T cells, T cell-derived IFNγ secretion increased (62), an observation that was also substantiated *in vivo* when irradiation of established B16F10 tumors resulted in an increase of IFNγ-producing spleen cells (63).

However, as mentioned, complete analysis of the effects of drug-treated cancer cells on DC maturation is limited to only few therapies. Other treatments are simply hypothesized or speculated to induce fully mature DC phenotype, but these are claims supported by only indirect data. **Table 1**, recapitulates the available information about DCs-stimulating capacities of anticancer treatments.

Besides these conventional/experimental anticancer treatments, it is also emerging that targeted therapies can induce cancer cell death, capable of affecting DC maturation status. One such therapy is Vemurafenib (PLX4032), the inhibitor of mutated $BRAF^{V600E}$ kinase, which is predominantly used in patients with melanoma. Incubation of cancer cells (that harbor $BRAF^{V600E}$ mutation) with iDCs followed by poly(I:C) stimulation of the latter, down-regulated the release of TNF and IL-12 (IL-12 being crucial for effective functional maturation of DCs) (64). However, when cancer cells were pre-treated with Vemurafenib, the release of TNF and IL-12 from poly(I:C) matured DCs was re-established to a level obtained in the control (matured DCs without cancer cells) (64). Moreover Vemurafenib is known to increase TAA levels, such as MART1 and gp100 (65).

In conclusion, in future it is necessary to find and test more ICD inducers in order to better understand the diversity that fully mature DCs may exhibit in terms of activation characteristics. Also, it would be necessary to (re-)analyze certain existing therapies for their potential to cause DC maturation irrespective of whether they induced ICD.

SEMI-MATURE DCs

In the literature, evidence indicates that some anticancer treatments may cause "moderate" stimulation of an immune response. Under such circumstances the immune system activating signals are not strong enough or not persistent enough to establish a stable anticancer immunity. For DCs, this means that these APCs lack either the required phenotypic maturation markers and thereby are not capable to efficiently interact with T cells, or the required signature cytokine pattern released from loaded DCs and ultimately resulting in "immunocompromising" actions. Tolerogenicity induced by semi-mature DCs is connected with release of immunosuppressive cytokines like IL-10, TGF-β (66), plasma membrane expression of programed cell death ligands, like PD-L1 or PD-L2 (67), and with stimulation of T_{regs} expansion (67).

Phenotypically mature DCs

A good example of a treatment that induces phenotypic maturation of DCs, independent of the immunostimulating profile of cytokines is bevacizumab. This epidermal growth factor receptor (EGFR)-blocking antibody, which blocks angiogenesis, only induced phenotypic maturation of DCs upon their co-incubation with treated cancer cells (68). Nevertheless it should be highlighted that, on the one hand, addition of bevacizumab to co-cultures resulted in increased IL-6, but decreased IL-12 release (68). Moreover, it was shown that bevacizumab-treatment of patients with metastatic colorectal cancer increased total lymphocyte number (69) and had the potential to increase extravasation of T cells into the tumor bed, previously observed for the therapeutic paradigm of anti-EGFR antibody combined with adoptively transferred T cells in mice models (70).

Furthermore, cetuxinib, another EGFR-blocking antibody that prevents signaling from growth factors, shows similar results. Despite the fact that colon cancer cells treated *in vitro* with cetuxinib were phagocytosed by iDC (71) and induced the up-regulation

of maturation markers (72), there is no investigation, till now, of the cytokines required for characterization of maturation status of these DCs. Thus this treatment should not be incorporated into the group of therapies that induce "full mature DCs" – as of now. Nevertheless, cetuximab treatment has other features that demonstrate its positive effect on the immune system: its capacity to stimulate NK cell mediated antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, are well documented (72).

Finally sunitinib (an inhibitor of receptor tyrosine kinase)-treated melanoma cells enhanced the maturation status of DCs (measured by the percentage of $CD86^+$ cells). However no investigation of DC-secreted cytokines has been performed. In spite of this, one can be relatively positive about its immunoinhibitory effects, as cytotoxic T lymphocytes (CTLs) incubated with these loaded DCs did not increase their secretion of IL-6 (73).

Thus, while in some cases, certain therapeutic treated cancer cells induce formation of semi-mature DCs, yet for others the indications in this direction are either mixed or poorly studied. More analysis is required on the cytokine levels to ascertain whether such therapeutics are able to cause formation of semi-mature DCs or not. Last but not least, it is also necessary to analyze further the direct effects of anticancer treatments on DCs maturation (in set-ups where these therapies are not intended to directly affect the DCs) – an aspect that has received the least attention in studies addressing DC-based immunity.

DC-BASED CANCER IMMUNOTHERAPY

Most of the clinically used anticancer therapies if systemically administered strongly affect not only cancer cells but as well the cells from tumor microenvironment, systemic hematopoietic cells, and rapidly dividing bone marrow cells. Despite the fact that recently platinum-based drugs, at clinically applicable concentrations, have been shown to enhance cytokine-induced DC maturation *in vitro* (74), vast majority of the effects on the non-cancer cells are of negative nature (i.e., prevention of differentiation of new immune cells from progenitor bone marrow cells and lymphopenia or leukopenia). These actions reduce the number of immune cells capable of sensing the danger and immune-stimulating signals released by dying cancer cells thereby compromising anticancer immunity. To evade this effect, a DC-based immunotherapy approach can be employed in a couple of ways: (1) by directly targeting/stimulating the DCs *in vivo* so as to accentuate their anticancer phenotype or (2) by stimulating the DCs *ex vivo* and infusing them back into the host for carrying out anticancer effector function.

Starting from 1998 there were few trials testing the *in vivo* DCs' stimulation with synthetic peptides (75–77). Most of them however failed as they were unable to effectively stimulate $CD4^+$ cellular responses (75, 78, 79) and stimulation of Th2 type cytokines (80, 81). Learning from the abovementioned studies, Walter *et al.* showed that patients pre-treated with single-dose cyclophosphamide as well as vaccinated with TAAs peptides and granulocyte macrophage colony stimulating factor (GM-CSF), showed clinical responses in Phase I and II trials (82). To further improve the peptide/protein anticancer vaccines the idea of combining TLR agonist administration with the vaccines emerged. The idea was taken up by GlaxoSmithKline that invented AS15 adjuvant that combines TLR4 and TLR9 agonists (83). Patients with MAGE-$A3^+$ melanoma administered with MAGE-A3 peptide in combination with AS15 in Phase II trial (NCT00086866 and NCT00290355) showed clinical activity (84). The study is being followed up by a Phase III trial.

An alternative, to direct *in vivo* DCs' stimulation is, isolation of DCs' precursors from the patient (through leukapheresis) and maturation/stimulation of these precursors *ex vivo* followed by allogeneic injection of these fully mature DCs back into the patient. Nowadays there are various ways applied to generate cancer cells-specific DCs: the stimulation can be done with specific TAAs (full length or short peptides), tumor lysates (freeze-thawed or acid eluted), electroporation/transfection of DCs with total cancer cell-mRNA, creating DC-cancer cell fusions, or with tumor derived exosomes (TDEs) (as discussed below). Alternatively DCs can also be genetically manipulated to express specific TAAs. Moreover as the stimulation is performed *ex vivo* there is a possibility to additionally co-stimulate with cytokine "cocktails" to assure their strong maturation. For example in 2010 a Provenge treatment strategy on similar lines got approved by FDA for therapy of patients with castration-resistant prostate cancer (85). The treatment consists of *ex vivo* stimulation of DCs with PA2024 that is a fusion protein of prostatic acid phosphatizes (TAA present in 95% of this type of tumor) and GM-CSF. The Phase III clinical trial revealed increased overall survival of patients treated with Provenge in comparison to placebo (86, 87).

Currently, there are many Phase I, II, and III clinical trials that test the effect of different anticancer DC vaccination strategies on various cancer types. The running/finalized clinical trials were recently thoroughly summarized by Galluzzi *et al.* thus we refer readers interested in this topic to "Trial Watch" publication (88).

EXOSOMES; AS LONG DISTANCE MESSENGERS, MODULATORS, OR SUPPRESSORS OF DC-ASSOCIATED ANTICANCER IMMUNITY?

Phenotypic maturation and functional stimulation are well-established markers of DC maturation as well as the ability of DCs to "prime" anticancer immune responses (9). Modulation of these two relevant DC-associated biological parameters by cancer cells (on the levels of TAAs, DAMPs, or danger signals and cytokines/chemokines) is considered to make the difference between immature, semi-mature, and mature DCs (2, 7, 35, 40), as discussed above. However, depending on the anticancer therapy under consideration, DC markers and cancer cell-based modulators sometime fail to completely account for the observed failure of or reduction in anticancer immunity (89). Thereby these may point toward other DC or cancer cell-based autocrine or paracrine modulators of immunity which are capable of transmitting signals (21).

One vehicle type capable of long distant transport of cellular material are the endosome-derived nano-vesicles, known as exosomes (90). These vesicles are derived by inward budding of the multi-vesicular body membrane and have been implicated in cell-cell communication (91). Historically, exosomes were classified as a simple mechanism for the removal of unwanted cellular material (92, 93), yet more recently they have been implicated in the transmission of signals between cells, both locally and over

long distances, effecting cells of different lineages, demonstrating the capacity to influence cellular signaling, and outcomes of stress responses (94–96). Where the physiological outcome depends on both the type of cell the exosome originates from and the type of stress the cell is exposed to, known to alter protein and lipid signatures in a context-dependent manner.

Certain cancer cells are known to exaggerate their generation of exosomes, demonstrating constitutive release, delivering tumor derived signals throughout the local tumor microenvironment and beyond within various body fluids (97, 98). These signals have been implicated in the transmission of pro-tumorigenic, angiogenic, and metastatic signals, as well as factors capable of stimulating/inhibiting immune responses (97, 99, 100). Here we will focus on the dynamic relationships that exist between the signals released or received between cancer cells and DCs and highlight key components that may sway the outcome in the context on anticancer immunity.

THE EFFECT OF CANCER-DERIVED EXOSOMES ON DENDRITIC CELLS

Antigen acquisition by DCs is an essential step in the induction of antigen-mediated immune responses. These antigens can be sequestered by DCs in the form of infectious agents, dying infected cells or in the case of tumors, by the engulfment of dying cells or exosomes that are secreted by living/stressed or dying cells. As the protein signature of an exosome is dependent on the cell of origin as well as their viability, TDEs are abundant in TAAs (Her2/Neu, MART1, TRP1/2, gp100) (101), antigen-presenting molecules (MHC class I, heat-shock proteins) (102), as well as varying tetraspanins (such as CD81) (103–105). These privileged carriers of antigens and immunostimulatory molecules exposed on exosomes have been shown to activate DCs (101). Research identified that exosomes could induce phenotypic and functional maturation of DCs, demonstrating enhanced cell surface expression of MHC-II, CD80, CD86, and CD40 as well as increased IL-12p70 production (106). For example, melanoma exosomes were shown to deliver MART1 tumor antigens to monocyte-derived DCs, allowing for successful cross-presentation (101). Moreover, *in vivo* assessment of TDEs capacity for immunomodulation demonstrated their potential to prevent autologous tumor development, in a CD4/CD8-dependent manner (107). TDE mediated DC maturation and antigen presentation (MHC-II and ICAM) propagates T cell stimulation, demonstrated by increased $CD4^+$ and $CD8^+$ T-cell proliferation, the induction of enhanced CTL based tumor cell lysis (108, 109) and the generation of Th1-type memory (110). Moreover, exosomes derived from DC cells exposed to TAAs demonstrate 50-fold higher efficiency and 3-fold higher T cell activation potential than non-TAA exposed controls (109).

Conversely, other studies have demonstrated the immunosuppressive nature of TDEs. Work into the role of TDEs highlighted their tumor suppressor potential (111), however the majority of data, till now, indicates a more potent immunosuppressive nature. For example, TDEs can prevent DC differentiation *in vitro*, in such a manner that a pool of $CD14^+$ HLA-$DR^{neg/low}$ cells was generated, culminating in the marked reduction of autologous T cell stimulation (112). Also, *in vivo* experiments demonstrated an accumulation of undifferentiated myeloid cells in the spleen of mice after TDEs administration, consequently resulting in the formation of a DC population that was incapable of maturation (99). Furthermore, this inhibition of DC maturation/differentiation was also observed in human monocytes, following exposure to TDEs (99). Moreover, TDEs have the potential to activate myeloid derived suppressor cells (MDSCs), hampering immune responses, in this case *via* T_{regs} (113).

The potential for TDE to influence an immune response has generated contrasting bodies of research. However these observations may both be true and simply a consequence of experimental design. For instance, time is an important issue for response outcome when the TDE interact with immune cells. Yu *et al.* investigating the effect of TDE on bone marrow derived myeloid precursors, described a significant reduction in DC differentiation, induced by treatment with GM-CSF, when the exposure occurred within 3 days (99). In contrast, Andre *et al.* showed that pulsing iDCs with TDE, after 5 days of GM-CSF treatment, resulted in an observed DC-mediated T cell response (101). Moreover, research into the effect of TDE, on induction of cytokine release from monocytes, demonstrated that a cacophony of pro-inflammatory cytokines (such as TNF, IL-6), as well as immunosuppressive factors (such as IL-8, IL-10, TGF-β) (114) were released. Importantly immunosuppression was predominantly mediated *via* TGF-β. The ability of TDE to induce IL-6 expression and/or release has been implicated in their inhibition of myeloid precursor differentiation, as well as accentuating the immunosuppressive capacity of MDSC, which were themselves activated by TDEs (99). Furthermore, research demonstrated enhanced exosomal HSP72, induced by IFNγ stimulation of tumor cells, resulted in the up-regulation of CD83 and potentiation of IL-12 production in DCs (115).

Alterations in cancer dendritic cell-derived exosome (DEX) expression of key immune-modulators have been shown to be evoked by both tumor microenvironmental stress as well as cellular stress induced by anticancer therapies, both traditional and targeted approaches (101–105). However, due to the vast number of cell types that excrete exosomes, little is known about the effect of therapy specifically on exosome-based host immune activation in clinical settings. However, research *in vitro* has demonstrated significant enrichment of TAAs as a consequence of therapy. Moreover, combination therapy with exosomes and DNA alkylating agents (such as cyclophosphamide) significantly potentiated cancer killing compared to single agent (117, 118). Fortunately, due to the biomarker potential of exosomes, progress into exosomal population isolation is allowing further investigations of the immunomodulatory and overall clinical potential of TDEs (116).

Importantly, the mode of antigen secretion can also alter the immunogenicity toward TAAs (119). Antigens loaded into nanovesicles were shown to incite a significantly stronger immune response, than when the same antigens were secreted freely. Therefore the manipulation of how antigens are presented to immune cells may be used to enhance the success of anti-tumor vaccinations (107). So, due to the contradictory effects of TDE on DC-induced immune responses, the concept of TDE as a targeted-cancer therapy was quickly surpassed by the use of safer and more focused DC-DEXs, loaded with TAAs (120, 121).

THE EFFECT OF DENDRITIC CELL-DERIVED EXOSOMES ON CANCER CELLS

The potential for endogenous DEXs to induce anticancer responses remains unclear. However, existing research has identified that DEXs express, on their surface, multiple TNF superfamily ligands (122). Through these ligands they can incite anticancer immunity directly *via* the induction of cancer cell apoptosis, as well as indirectly through the activation of NK cells (122, 123). Recent work shows that similar to DCs, DEXs contain TNF, FasL, and TRAIL. These ligands have the potential to trigger caspase activation and apoptosis in a tumor cell models (122). Moreover, DEXs can also activate NK cells and stimulate their IFNγ secretion, inciting immune responses (122, 123).

However, research over the past decade has highlighted more the use of engineered DEX as a feasible and successful route to activate anti-tumor modalities *in vivo* (123, 124), that has gone on to demonstrate success clinically (120). Interestingly, treatment with engineered DEXs has shown a stronger anticancer effect than the use of the DCs they are derived from to re-activate downstream immune responses. These observations may in part be explained by the immunosuppressive effect of the tumor microenvironment on DC phenotypic functionality (125). Zitvogel and colleagues demonstrated a perturbation in growth of mastocytoma and spontaneous mammary carcinoma tumors by day 10, following inoculation with DEXs, derived from bone marrow DC that were pulsed with acid eluted tumor antigens (107). Furthermore, by day 60 ∼50% of mice treated with DEXs were diagnosed tumor free (107). Interestingly, when re-challenged, the mice demonstrated tumor rejection unless inoculated with a differing cancer type, implying long-lasting anti-tumor immunity stimulated by DEXs (107). Furthermore, Taieb *et al.* investigating the combination of DEXs with cyclophosphamide showed that DEXs were capable of boosting the immune response toward immunogenic cancers, showing synergistic tumoricidial potency toward pre-established tumors (118).

Elegant research into the potential of DEXs as anticancer modulators demonstrated that DEXs harvested from bone marrow derived DCs that had been stimulated by LPS treatment mature dendritic cells derived exosomes (mDEXs), compared to untreated immature dendritic cells derived exosomes (imDEXs), were significantly enriched in molecules (such as ICAM-1) capable of mediating T cell priming, enhanced T cell proliferation and the stimulation of naïve T cells to differentiate and produce cytokines (108). The research of Naslund and colleagues showed that DEX treatment induced T cell responses, yet in a B cell-dependent manner (126). This suggests that immunization with DEXs carrying only peptides for T cells would induce a sub-optimal response (126). Furthermore, protein-loaded rather than peptide-loaded DEXs showed greater T cell responses *in vivo* and a superior anti-tumor capacity (126). Interestingly, the induced T cell response requires the presence of B cells and mice deficient in complement activation and antigen shuttling by B cells had reduced DEXs-induced responses (126). Solidifying the dynamics of exosomal signaling in immune cell activation and anti-tumor immunity, DEXs secreted into the extracellular milieu during cognate T cell–DC interactions, are targeted and engulfed specifically by T cells, via the leukocyte function-associated antigen-1 (LFA-1) receptor (127).

Moreover, findings from preliminary Phase I clinical trials for the use of DEXs as a treatment for stage IV melanoma and non-small cell lung cancer, demonstrated a restoration of NK cell activity in over 50% of patients (107, 120). This increase in NK cell activity was shown to stimulate their cell killing capacity *in vitro* (120). Therefore, their lipid composition, that itself possesses adjuvant qualities and exosome stability within the circulation (128–130), coupled with simultaneous expression of MHC class I and II molecules, as well as a plethora of co-stimulatory molecules (102, 131), may indicate the cocktail of requirements that deem DEXs capable to incite anti-tumor or pro-immunogenic effects. Furthermore, the reported lack of toxicity highlights DEX-based therapies as an interesting modality for cancer therapy (107, 120). Further to this, investigation on combination of DEX-targeted therapies with traditional therapeutics or other modern targeted approaches should be done to explore their potential to restore immune activity in the fight against cancer.

CONCLUDING REMARKS

The induction of an efficient anticancer immune response is thought to contribute to the success of anticancer treatments, by the establishment of a robust T cell mediated response capable of sustaining long-term control of cancer. Upon activation, DCs are crucial inducers of T cell immunity and are therefore at the frontline of immune-regulated responses. Hence, triggering proper maturation of DCs is an outstanding therapeutic objective as it may boost anti-tumor immunity and thwart cancer-induced immunosuppression. The discovery of different DCs sub-populations that exhibit wide functional plasticity has made the initial dichotomy between immature/tolerogenic and mature/immunogenic DCs, obsolete. However, in spite of a functional definition of these DCs phenotypes, which ranges from tolerogenic, partial/semi-mature to fully mature DCs, it still remains challenging to understand how, when, and to what extent this dynamic spectrum of DC activation drives tumor-specific tolerance or anti-tumor immunity, also in the context of anticancer therapy. In this respect, the existing (mostly immunosuppressive) or therapy-generated tumor microenvironments and the cross-talk between (dying) cancer cells and DCs, established through soluble (cytokines/chemokines) and vesicular (exosomes) mediators, are emerging as crucial determinants of DC maturation status and anticancer immune responses. Future preclinical research combined with clinical investigations, will disclose whether therapeutics inducing immunogenic cancer cell death, will meet the therapeutic objective of re-establishing the proper interface between dying cancer cells and DCs, promoting their fully mature/immunogenic status that is required to sustain anti-tumor immunity.

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Impact of MAPK pathway activation in BRAF^{V600} melanoma on T cell and dendritic cell function

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Constitutive upregulation of the MAPK pathway by a BRAF^{V600} mutation occurs in about half of melanomas. This leads to increased oncogenic properties such as tumor cell invasion, metastatic potential, and resistance to apoptosis. Blockade of the MAPK pathway with highly specific kinase inhibitors induces unprecedented tumor response rates in patients with advanced BRAF^{V600} mutant melanoma. Immune checkpoint blockade with monoclonal antibodies targeting cytotoxic T-lymphocyte antigen 4 and programed death-1/PD-L1 has also demonstrated striking anti-tumor activity in patients with advanced melanoma. Tumor responses are likely limited by multiple additional layers of immune suppression in the tumor microenvironment. There is emerging preclinical and clinical evidence suggesting that MAPK inhibition has a beneficial effect on the immunosuppressive tumor microenvironment, providing a strong rationale for combined immunotherapy and MAPK pathway inhibition in melanoma. The T cell response has been the main focus in the studies reported to date. Since dendritic cells (DCs) are important in the induction of tumor-specific T cell responses, the impact of MAPK pathway activation in melanoma on DC function is critical for the melanoma directed immune response. BRAF^{V600E} melanoma cells modulate DCs through the MAPK pathway because its blockade in melanoma cells can reverse suppression of DC function. As both MEK/BRAF inhibition and immune checkpoint blockade have recently taken center stage in the treatment of melanoma, a deeper understanding of how MAPK pathway inhibition affects the tumor immune response is needed.

Keywords: melanoma, dendritic cell, T cell, BRAF, MEK, immunotherapy, kinase inhibitor

INTRODUCTION

Melanoma incidence rates have been increasing for at least 30 years. It is estimated that 76,690 individuals will be diagnosed in 2013 (1). The disease is usually curable when detected in its early stages (thin primary tumor, no lymph node involvement). For patients with unresectable or metastatic melanoma, recently emerged novel systemic treatment modalities such as Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and Programed Death-1 (PD-1)/PD-L1 blockade as well as BRAF and MEK inhibition have expanded the spectrum of therapeutic options (2–13). The successes with immune checkpoint blocking antibodies in the treatment of patients with metastatic melanoma, with reported response rates of up to 50% are remarkable. Both CTLA-4 and PD-1/PD-L1 blockade can induce long lasting tumor responses in the absence of vaccination, suggesting that endogenous tumor-specific T cells exist in a substantial proportion of patients and that these T cells, once uncoupled from the inhibitory effect mediated by CTLA-4 and/or PD-1/PD-L1 can mediate effective tumor cell lysis (2, 3, 12–15). Multiple other immune suppressive mechanisms are at work in the tumor environment, including additional inhibitory molecules such as Tim-3 (16) and LAG-3 (17), regulatory T cells, myeloid derived suppressor cells (18), and soluble immunosuppressive mediators such as IDO (indoleamine 2,3-dioxygenase), arginase, prostaglandin E2 (PGE2), IL-6, IL-10, VEGF, TGF-β along with other suppressive cytokines and chemokines. Given the multitude of suppressive mechanisms, it is remarkable that a relatively high proportion of patients can achieve objective tumor responses by blockade of a single pathway, such as PD-1/PD-L1 or CTLA-4.

Approximately half of melanomas harbor a somatic point mutation of the BRAF oncogene at codon 600 (V600E and V600K). This mutation results in constitutive activation of the MAPK pathway and increased oncogenic behavior mediated through a variety of mechanisms such as increased apoptosis, invasiveness, and metastatic potential. The MAPK pathway is an important therapeutic target in melanoma: BRAF, MEK, and combined BRAF/MEK inhibition with small molecule kinase inhibitors are successful treatment strategies in patients with BRAF mutant metastatic melanoma (6–10). However, resistance to these treatments develops almost universally, limiting the median duration of treatment responses to 6–9 months. Investigation of resistance mechanisms and potential strategies to overcome resistance is a very active area of research; a number of different mechanisms have been identified, including the reactivation of MAPK signaling by other pathways (19–24).

Given the treatment successes with both kinase inhibition and immune checkpoint blockade in melanoma, there is considerable

interest in combinatorial approaches. The promise is to combine the response durability that is characteristic for patients responding to immunotherapy with the high response rate seen with BRAF inhibition. The scientific rationale for such strategies is based on the interplay of the MAPK pathway and the tumor immune response in the microenvironment. Activation of signaling pathways in tumor cells have long been implicated in promoting suppressive immune networks in the tumor environment (25, 26). There is emerging evidence of a link between the MAPK pathway in melanoma and the tumor immune response. Preclinical and clinical observations indicate that inhibition of the MAPK pathway may have a favorable effect on the melanoma-specific immune response on the level of T cells, tumor cells, stromal cells, and dendritic cells (DCs) (**Table 1**; **Figure 1**).

BRAF AND MEK INHIBITION IN MELANOMA CELL LINES LEADS TO UPREGULATION OF TUMOR ANTIGENS AND INCREASED RECOGNITION BY MELANOMA-SPECIFIC T CELLS *IN VITRO*

In melanoma cell lines, MEK and BRAF inhibition leads to increased expression of melanoma differentiation antigens (MDAs) such as gp100, MART-1, and tyrosinase on the mRNA and protein levels (27–29). The underlying mechanism of oncogenic BRAF-regulated MDA expression is unclear. It has been suggested that oncogenic BRAF suppresses MDA expression through microphthalmia-associated transcription factor. However additional pathways are likely involved and may account for the heterogeneity of MDA induction observed across different cell lines including mutant and wild-type cell lines (30). Increased expression of gp100 and MART-1 leads to improved antigen recognition by T cells as measured by IFN-γ production (27). Upregulation of gp100 and MART-1 was seen in both BRAF mutant and WT melanoma cell lines. BRAF inhibition did not negatively impact lymphocyte function, whereas MEK inhibition negatively affected T cell proliferative potential, viability, and IFN-γ production. These data were recently confirmed *in vivo* in patients with metastatic melanoma (31). Increased MART, TYRP-1, TYRP-2, and gp100 expression was found in metastatic melanoma specimens obtained from patients after treatment with BRAF and/or MEK inhibition. Interestingly, melanoma antigen expression in metastatic tumors was decreased at the time of tumor progression in patients treated with a BRAF inhibitor and partially restored upon initiation of dual MEK and BRAF blockade.

INCREASED FREQUENCY OF TUMOR INFILTRATING LYMPHOCYTES AFTER BRAF INHIBITION

In an adoptive T cell transfer (ACT) model, frequencies of gp100 specific luciferase expressing pmel-1 T cells were markedly increased in gp100 expressing melanoma lesions after treatment with vemurafenib (32) and this was associated with improved tumor response compared to either vemurafenib or ACT alone. This observation was specific to BRAF mutant tumors and independent of BRAF inhibition-mediated upregulation of MDA. In this model, the increased intra-tumoral T cell frequencies were attributed to decreased VEGF in the tumor. It was previously shown that VEGF/VEGFR-2 inhibition can upregulate endothelial adhesion molecules in tumor vessels, which can in turn increase the infiltration of leukocytes in tumors (33). Wilmott et al. confirmed the observations of increased intra-tumoral T cell frequencies in melanoma patients who were treated with a BRAF inhibitor (34). Increased frequencies of CD4 and CD8 cells were seen in both intratumoral and peritumoral regions of metastatic tumor specimens obtained between 3 and 15 days after treatment initiation. The increase in lymphocyte numbers inversely correlated with tumor size, but not with clinical objective responses. Notably, intra-tumoral lymphocyte frequencies returned to pre-treatment levels at the time of tumor progression. Similar increases in tumor infiltration by CD8 cells (but not CD4 cells) and decrease upon tumor progression in melanoma patients treated with BRAF or dual BRAF/MEK inhibition was reported by Frederick et al. (31).

MAPK INHIBITION AFFECTS T CELL FUNCTIONALITY AND SECRETION OF IMMUNOSUPPRESSIVE CYTOKINES IN THE TUMOR MICROENVIRONMENT

In an ACT model using the murine BRAF^{V600E} mutant melanoma SM1 and transgenic T cells recognizing gp100 and ovalbumin (OVA), combined ACT and vemurafenib induced superior anti-SM1 tumor immune responses compared to either of the therapies alone. In this study, no difference in frequencies of adoptively transferred T cells was observed in tumors, lymph nodes, or spleen as assessed *ex vivo* by flow cytometry and immunofluorescence imaging and *in vivo* by tracking of the firefly luciferase transgene-labeled T cells using bioluminescence imaging when mice were treated with vemurafenib in addition to ACT. However, adoptively transferred T cells exhibited increased functionality as measured by IFN-γ production and their ability to lyse tumor cells (35) in mice treated with ACT and vemurafenib.

CROSS-TALK BETWEEN THE MAPK PATHWAY IN BRAF MUTANT MELANOMA AND DCs

Sumimoto et al. demonstrated that BRAF^{V600E} mutant cell lines can produce immunosuppressive cytokines such as VEGF, IL-6, and IL-10 and that MEK inhibition with U0126 and BRAF inhibition using BRAF^{V600E} specific RNAi suppressed secretion of these cytokines. IL-12 and TNF-α production by DCs exposed to supernatant from the BRAF mutant A375 melanoma cell line prior to maturation by LPS was suppressed (36). This inhibitory effect was mediated by IL-6, IL-10, and VEGF and could be partially reversed by pre-treatment of the melanoma cells with BRAF^{V600E} specific RNAi, indicating that constitutive activation of the MAPK pathway in melanoma cells may lead to compromised DC function and that this immune evasion may be overcome by MAPK inhibition. In a separate study, IL-10 expression in the melanoma line A375 was found to be induced by TGF-β, an effect that was mediated by cross-talk between the Smad, PI3K/AKT, and MAPK pathways (37).

We recently explored a potential link between constitutive MAPK pathway upregulation driven by a BRAF^{V600} mutation and DC function using a human melanoma-DC co-culture system

Table 1 | Effects of MAPK inhibition on immune function and melanoma.

Study type	Model	Immune cell type studied	Effect of MAPK inhibition	
			Immune cells	**Melanoma cells**
In vitro (human) (38)	Monocyte-derived moDC co-cultured with BRAF^{V600E} mutant and WT melanoma cell lines DC maturation with Poly-ICLC	DCs	Restored IL-12 and TNF-α production by DCs exposed to BRAF mutant melanoma cells treated with MEK and BRAF inhibition	No consistent suppression of cytokine production observed
In vitro (human) (36)	Monocyte-derived moDC cultured with supernatants of BRAF^{V600E} mutant melanoma cell lines DC maturation with LPS	DCs	Restored IL-12 and TNF-α production by DCs exposed to supernatants of melanoma cells treated with BRAF^{V600E} – specific RNAi	Suppression of IL-6, IL-10, and VEGF secretion
In vitro (human) (27)	BRAF^{V600E} mutant and WT melanoma cell lines treated with MEK and BRAF inhibition. Melanoma cells cultured with TCR-transgenic CTL specific for gp100, MART-1	CTL	Increased IFN-γ production by melanoma-specific CTL cultured with BRAF^{V600E} melanoma upon MEK and BRAF inhibition	Increased expression of MDA
In vitro (human) (42)	Mixed lymphocyte reaction with DCs, PBMCs, and T cells	DCs, T cells	Suppressed T cell activation by DCs exposed to melanoma overexpressing CD200; effect abrogated by CD200 knockdown with shRNA	Not assessed
Mouse adoptive T cell transfer (35)	BRAF^{V600E}-driven murine model of SM1 melanoma Adoptive transfer of C57BL/6 mice with TCR-transgenic lymphocytes	OVA and pmel-1 TCR-transgenic lymphocytes	No effect on expansion, distribution, or tumor accumulation of adoptively transferred T cells Increased T cell functionality (IFN-γ production, intrinsic tumor cell lysis)	No effect on gp100 expression on SM1 cells Increased tumor response with BRAF inhibition + adoptive T cell transfer
Mouse adoptive T cell transfer (32)	Xenograft with gp100 expressing melanoma cell lines. Adoptive transfer of C57BL/6 mice with TCR-transgenic gp100-specific pmel-1 T cells	Pmel-1 TCR-transgenic T cells	Enhanced infiltration of BRAF mutant, but not BRAF WT tumors with adoptively transferred T cells Increased VEGF production in tumors	Increased tumor response with BRAF inhibition + adoptive T cell transfer
Melanoma patients (34)		Intra-tumoral CD4 cells, CD8 cells, CD20 cells, Granzyme B, CD1a^{+} DC	Increased CD4 and CD8 cell frequencies in post-treatment tumor specimens Correlation between increased tumor CD8 infiltration and decreased tumor size and increase in tumor necrosis Occasional CD1 DCs present in post-treatment biopsies in 2 patients	Objective tumor responses on CT imaging
Melanoma patients (31)		Intra-tumoral CD4^{+} cells, CD8^{+} cells, IL-6, IL-8, IL-10, TGF-β, granzyme B, perforin, Tim-3, PD-1, PD-L1	Increased CD8^{+} cell frequencies No effect on CD4 cells Decreased IL-6 and IL-8 production Increased expression of Tim-3, PD-1, PD-L1 No effect on IL-10, TGF-β	Objective tumor responses on CT imaging Increased expression of MDA (MART-1, gp100, TYRP-1, TYRP-2)

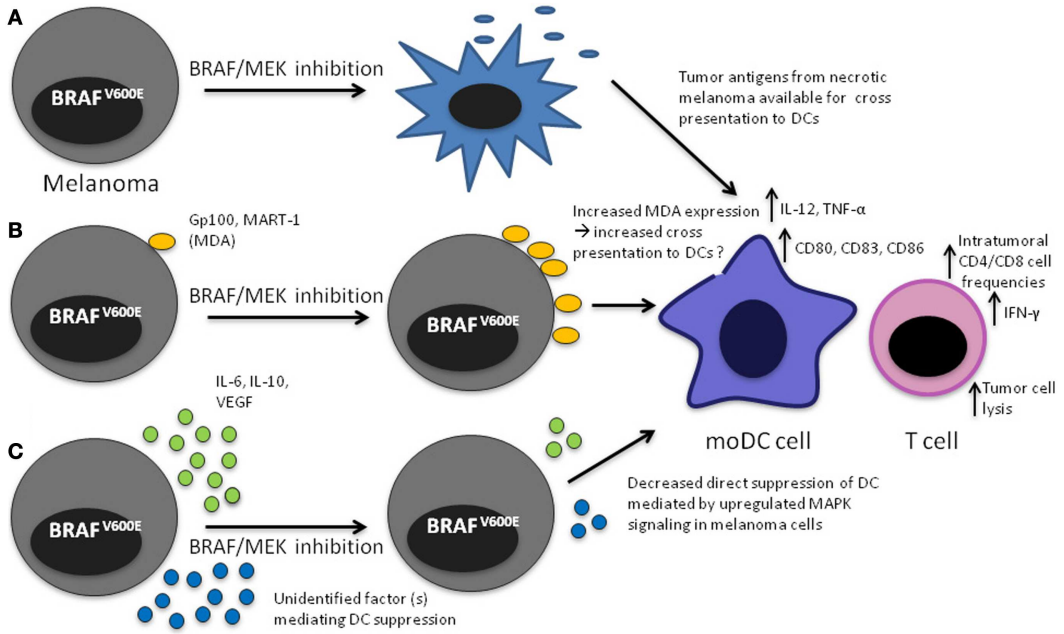

FIGURE 1 | Mechanisms that may lead to increased DC function upon MAPK pathway blockade in the tumor microenvironment. **(A)** Apoptosis/necrosis of melanoma cells results in release of tumor antigens that will presumably be available to DCs for cross presentation; **(B)** Increased expression of MDA through direct effect of MAPK pathway inhibition, potentially making them available to DCs for cross presentation, **(C)** decreased direct inhibition of DCs leading to increased IL-12 and TNF-α production.

(38). BRAF^{V600E} mutant and wild-type melanoma cell lines were treated for 24 h with the MEK inhibitor U0126, the BRAF inhibitor vemurafenib, or respective controls (U0124 or DMSO). After removal of supernatant, monocyte-derived immature DC from healthy donors were added, cultured for 24 h and then stimulated with poly-ICLC. Poly-ICLC was chosen as the DC maturation stimulus because it induces the secretion of proinflammatory cytokines in the absence of IL-10 and is a potent TLR3 and MDA5 agonist (39). It has been widely used as a cancer vaccine adjuvant in clinical trials. We found that IL-12 and TNF-α production by DCs was inhibited when DCs were exposed to melanoma cells treated with vehicle control. Notably, the secretion of both cytokines could be partially or completely restored with both MEK and BRAF inhibition in BRAF^{V600E} mutant, but not wild-type cell lines. Furthermore, CD80, CD83, and CD86 expression on DC was decreased upon co-culture with melanoma cells and could be partially restored with BRAF inhibition in BRAF^{V600E} mutant melanoma cell lines. The inhibition of IL-12 and TNF-α secretion by DCs was not cell-contact dependent. In contrast to the study by Sumimoto, a soluble factor responsible for mediating the suppressive effect could not be identified in our investigations. It is possible that continuous local production of small amounts of soluble mediators by melanoma cells in close proximity to DCs accounts for the inhibitory effect observed in the melanoma cell/DC co-culture experiments in our study.

CD200, a type I membrane-associated glycoprotein and member of the immunoglobulin superfamily is highly expressed on melanoma cells and was found to be regulated by ERK activation (40). CD200 mRNA expression levels were found to be positively correlated with tumor progression. Moreover, MEK inhibition with U0126 and knockdown of mutant BRAF resulted in reduced expression of CD200 mRNA in melanoma cell lines. Of note, through interaction with the CD200 receptor, which is expressed on macrophages and DC, CD200 mediates an inhibitory signal (41). In mixed lymphocyte reactions with T cells, DCs, and melanoma cells, T cells produced larger amounts of IL-2 when CD200 in melanoma cells was knocked down with shRNA specifically targeting the CD200 ligand (42). These data suggest a link between MAPK/ERK activation in melanoma and the ability of DCs to activate T cells.

DIRECT IMPACT OF MAPK INHIBITION ON DCs

Since there is a strong clinical interest in combined immunotherapy and BRAF/MEK inhibition in melanoma, the direct impact of MAPK pathway inhibition on immune cells is of great interest. BRAF inhibition, even at high concentrations, does not appear to directly compromise T cell function, and there is emerging data showing that low doses of RAF inhibition may even enhance T cell activation (43, 44). Furthermore, frequencies of DCs, monocytes, T cells, B cells, NK cells, and regulatory T cells in peripheral blood from metastatic melanoma patients were not affected by BRAF inhibition (45).

There is some controversy about the direct impact of signaling through the MAPK pathway on DC maturation. In LPS and TNF-α-matured DCs, MEK inhibition leads to upregulation of

co-stimulation molecules, increased IL-12 secretion and enhanced ability to activate T cells (46), whereas activation of ERK in DCs leads to immune suppression, mediated by TGF-β and Treg cells (47). Only minimal or no effect of MEK inhibition on DC function was shown in other studies (48–51). Differences in the maturation stimuli may account for some of the inconsistencies observed in these investigations.

In monocyte-derived DC from healthy donors, MEK inhibition lead to reduced IL-12 and TNF-α secretion, whereas BRAF inhibition had no effect on cytokine production over a wide range of doses (38). The expression of CD40, CD80, CD83, and MHCI was also reduced by direct MEK inhibition, whereas it was unaffected by BRAF inhibition. In addition, DC viability was reduced with MEK, but not BRAF inhibition and the ability of DCs to induce T cell proliferation in an MLR was reduced with MEK, but not BRAF inhibition. The impact of MEK inhibitors currently used in the clinic on APC function *in vivo* remains to be determined.

MAPK INHIBITION MAY ENHANCE DC FUNCTION IN THE TUMOR MICROENVIRONMENT BY SEVERAL MECHANISMS

RESTORATION OF DC FUNCTION COMPROMISED BY MELANOMA CELLS

Our studies and earlier investigations by Sumimoto suggest that suppression of IL-12 and TNF-α production by DCs in the tumor microenvironment of a BRAF^{V600} mutant melanoma is mediated at least partially by constitutive activation of the MAPK pathway. These data also indicate that BRAF and MEK inhibition, by blocking the MAPK pathway in melanoma cells and thereby restoring IL-12 and TNF-α production in DCs, leads to improved DC function, presumably leading to better activation of melanoma-specific T cells. Notably in our studies there was none or only minimal apoptosis in BRAF^{V600E} mutant and WT melanoma cell lines after 48 h of MEK or BRAF inhibition. This is consistent with prior studies showing an anti-proliferative effect, rather than apoptosis, during the first few days of treatment with these kinase inhibitors (52, 53). It is therefore unlikely that the reversal of compromised DC function mediated by melanoma cells in the *in vitro* experiments is mediated by melanoma cell death. Similarly, in the experiments by Sumimoto, no significant cell death was observed after treatment of the melanoma line A375 with the MEK inhibitor U0126, indicating that decreased IL-10, IL-6, and VEGF production was a direct effect of MAPK pathway inhibition rather than mere death of the melanoma cells. Taken together, these data suggest that MAPK pathway activation in BRAF^{V600} mutant melanoma cells has a direct suppressive effect on the capacity of DC to activate T cells.

INCREASED CROSS PRESENTATION OF OVEREXPRESSED MELANOMA DIFFERENTIATION ANTIGENS BY DCs IN THE TUMOR OR LYMPH NODE?

Inhibition of the MAPK pathway in BRAF mutant melanoma leads to increased expression of MDAs (gp100, Mart-1, Tyrp-1, and Tyrp-2), resulting in improved antigen-specific recognition by gp100 and MART-1 specific TCR-transgenic CTL as measured by increased IFN-γ production *in vitro* (27). In patients with BRAF^{V600} mutant metastatic melanoma, MART-1 expression was upregulated in metastatic tumors after treatment with BRAF inhibition. Increased infiltration of metastatic tumors with both CD4 and CD8 cells in one study, and of CD8 cells, but not CD4 cells in another study was observed after treatment with BRAF inhibition. A correlation between intratumoral infiltration with CD8 cells and tumor necrosis was found in post-treatment biopsies in one study (34). In addition to the direct effect on CTL function shown *in vitro*, it is possible that MDA overexpression on melanoma cells in the tumor *in vivo* may lead to increased cross presentation of these antigens to DCs and thus further enhance the tumor-specific T cell response.

INCREASED CROSS PRESENTATION OF TUMOR ANTIGENS DERIVED FROM APOPTOTIC TUMOR CELLS AFTER MAPK INHIBITION

As outlined above, in short-term (48–72 h) *in vitro* experiments using BRAF mutant melanoma cell lines, apoptosis or necrosis does not have a significant role in mediating the effects of melanoma MAPK pathway inhibition on DCs. BRAF inhibition does however eventually induce apoptosis and necrosis as evident by the fact that tumors shrink markedly in the majority of patients. Tumor necrosis/apoptosis likely leads to the release of antigens, which may be available for DCs either residing in the tumor or in draining lymph nodes to be taken up, processed, and cross-presented to T cells. Cross presentation may be one of the mechanisms mediating the synergy observed with ACT and BRAF inhibition in melanoma mouse models, although no direct evidence was provided in the reported studies (32, 35).

CONCLUSION

Constitutive upregulation of the MAPK pathway in BRAF^{V600} mutant melanoma appears to directly impact DC function as evident by partial restoration of IL-12 and TNF-α secretion upon treatment of melanoma cells with MEK or BRAF inhibition. These effects have so far been shown only *in vitro*. The beneficial effects of MAPK blockade on the tumor immune microenvironment shown *in vivo* in mouse models and melanoma patients argue for a broader impact of these treatments on the tumor-specific immune response, including increased T cell frequencies and improved function and changes in cytokine secretion patterns. Several mechanisms that may account for the improved immune response have been described such as increased MDA expression on melanoma cells and decreased intra-tumoral VEGF production, others remain speculative, such as increased cross presentation to DCs resulting from BRAF/MEK inhibition-mediated necrosis/apoptosis of melanoma cells. These observations reinforce the rationale for clinical trials assessing MEK/BRAF inhibition and immunotherapy in combination in patients with melanoma. Further studies are needed to delineate the phenotype and function of DCs in patients treated with BRAF/MEK inhibition. Because of superior efficacy and potentially improved tolerability, combined BRAF-MEK inhibition will likely replace BRAF inhibitor monotherapy. Defining the impact of both BRAF and MEK inhibition on the immune response will therefore be critical.

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Therapeutic implications of immunogenic cell death in human cancer

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Dendritic cells (DCs) are central to the adoptive immune response, and their function is regulated by diverse signals in a context-specific manner. Different DCs have been described in physiologic conditions, inflammation, and cancer, prompting a series of questions on how adoptive immune responses, or tolerance, develop against tumors. Increasing evidence suggests that tumor treatments induce a dramatic change on tumor-infiltrating lymphocytes and, in particular, on some DC subtypes. In this review, we summarize the latest evidence on the role of DCs in cancer and preliminary evidence on chemotherapy-associated antigens identified in human cancers.

Keywords: dendritic cells, T cells, chemotherapy, immunogenic cell death, tumor antigens

INTRODUCTION

Cancer is characterized not only by abnormal cell growth but also by increased and diverse modality of cell death, which is sensed by innate immune cells including macrophages and dendritic cells (DCs). Dying cells can trigger either tissue homeostatic clearance by macrophages or processing by DCs, which can integrate signals from dying cells for presentation to T cells in an immunogenic or tolerogenic manner. It is well known that antigen-specific $CD4^+$ and $CD8^+$ T cells are detected in advanced tumor stages and that adoptive T-cell transfer can be very effective in cancer therapy (1).

Dendritic cells are essential in priming T-cell responses upon the processing and presentation of both exogenous antigens, which are preferentially presented on major histocompatibility complex (MHC) class II molecules to $CD4^+$ T cells, and endogenous antigens, which are preferentially presented on MHC class I molecules to $CD8^+$ T cells. The capacity of DCs to present exogenous antigens derived from other cells (usually necrotic or apoptotic cells) or soluble antigens on class I molecules is defined as cross-presentation (2, 3). Different types of DCs have been described according to different parameters, including where they are located, the type of antigen they present, and their ability to present antigens to T cells (4). From a simplified view, DCs travel in periphery tissues in search of potential antigens that are derived from pathogen-infected cells (foreign antigens); cancer cells re-expressing developmental antigens, for which the immune tolerance is low; or cancer cells expressing mutated proteins as a consequence of the oncologic process. They are attracted in inflamed tissue by metabolic products of cell death such as ATP, or they can be guided by chemokines secreted by innate immune cells such as macrophages. During the journey, they are characterized as having a high phagocytic capacity and a low antigen-presenting capacity: this status is referred to as an immature state. Different stimuli associated with bacterial or viral infections or damage signals can then activate DCs. In lymphoid tissues, DCs present antigens to B and T cells to initiate an adaptive immune response, depending on the presence of mature signals that direct adoptive responses.

Pathogen-associated molecular patterns (PAMPs) were first postulated by Janeway (5) and then identified in different species including insects. At the core of Janeway's hypothesis was the idea that similar structures are shared by different pathogens and that immune receptors [pattern recognition receptors (PRRs)] expressed by several types of innate immune cells have evolved to recognize them. On the same line of reasoning, it became clear that immune cells can be activated by damage (danger) signals, which share the properties of being undetectable to immune sensors during physiologic processes and being detectable in cases of injury. With a few exceptions (e.g., the association of cervix tumors with the papillomavirus, or of hepatocellular carcinoma with hepatitis B or C viruses), most tumors deregulate cell life usually in the absence of a non-self signal however, they can activate immune responses through danger signals that are referred to as, in analogy with PAMPs, damage-associated molecular patterns (DAMPs).

Dendritic cells carry out several complex tasks including antigen sampling in the periphery, cell maturation in the spleen and lymph nodes, and the critical decision-making process between immunity and tolerance (lack of immune response). These tasks are executed by DCs through a remarkable plasticity and an ability to integrate signals from a variety of receptors sensing extracellular and intracellular environments. This sophisticated system likely evolved in vertebrates as a way in which to avoid autoimmune diseases mediated by adaptive immunity; however, it can

limit an effective immune response against tumors, which derive from the self.

HUMAN AND MOUSE DCs IN PHYSIOLOGY AND CANCER

Human and mouse DCs are classified as classical DCs (cDCs) and plasmacytoid DCs (pDCs) (6) and present different morphologies: pDCs are round shaped, whereas cDCs have dendrites, distinct membrane markers, and different functions, and derive from different precursors within the myeloid lineage. This intricately connected system has made it difficult to distinguish DCs from other myeloid cells. To resolve this issue in mice, DC subtypes have been characterized through genetic ablation of key genes, transfer of purified cells, and functional studies. Traditionally, cDCs have been identified in mice by CD11c expression (7). However, depletion of cells expressing this marker resulted in ablation of not only cDCs but also pDCs (8). To obtain a more precise picture of DC populations, lineage-specific transcription factors have been identified [reviewed in Ref. (4)]. Two transcription factors, Flt3 and Xcr1, are associated with murine DCs, but not with macrophages, which is in line with their function during DC development. However, expression of transcription factors can be tissue specific. For instance, Zbtb46 distinguishes cDCs from other myeloid and lymphoid cells, but it is downregulated after DC stimulation; it is also found on endothelial cells, early erythroid progenitors, and monocytes stimulated with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin 4 (IL-4). Mouse cDCs in lymphoid tissues are divided into $CD8^+$ and $CD4^+$ T cells and functionally classified according to antigen presentation on MHC class I to CD8 T cells and class II to CD4 T cells, respectively (4). Importantly, $CD8^+$ cDCs carry out the unique function of cross-presentation of exogenous antigens on class I molecules (2, 3). More recently, mouse cDC lineage has been further refined using expression history of DNGR-1 gene (9). Transfer of precursor DCs expressing DNGR-1 in mice depleted of myeloid cells leads to the development of cDCs but not to pDCs, as observed in the transfer of unfractionated precursor DCs.

In humans, myeloid cDCs can be categorized as $CD1c^+$ ($BDCA1^+$) and $CD11a^+$ $CD141^+$ ($BDCA3^+$) DCs. The latter cells have been considered equivalent to mouse $CD8^+$ DCs, particularly because they express the C-type lectin receptor CLEC9A, which mediates the uptake of necrotic or dead cells and the cross-presentation of the related antigens (10). However, recent evidence from a systematic study of DC populations showed that the functional specialization of human DCs is completely different from that of murine DCs (11). In contrast to the murine models, all human DC populations tested ($BDCA1^+$ or $BDCA3^+$ cDCs, and even $BDCA2^+$ pDCs) express similar functions including cross-presentation and capacity of antigen transfer from phagoendosomes into the cytosol. In addition, Toll-like receptors (TLRs) are expressed differently by human and mouse cDC populations: both human and mouse cDCs express TLR1, 2, 3, 4, 5, 6, and 8, whereas TLR11, TLR12, and TLR13 are expressed only by mouse cDCs, and TLR 10 is unique to humans (12).

Plasmacytoid DCs represent a small fraction of DCs and have a round shape that is similar to antibody-secreting plasma cells. Regarding surface markers, pDCs are distinguished from cDCs by the expression of B220, Siglec-H, and Bst2 in mice and of BDCA2 (CD303) in humans (4). In both humans and mice, pDCs express TLR7 and 9 (13, 14). TLR7, 8, and 9 belong to a functional subfamily and detect PAMPs in endosomal/lysosomal compartments following acidification [reviewed in Ref. (15)]. After exposure to synthetic TLR7 or TLR9 agonists [e.g., imidazoquinoline compounds or guanosine analogs for TLR7/8, cytosine-phosphorothioate-guanine-oligodeoxynucleotides (CpG-ODNs) for TLR9], pDCs secrete interferon alpha and proinflammatory cytokines (IL-8 and tumor necrosis factor alpha) and undergo maturation, a differentiation program characterized by upregulation of the costimulatory molecules CD80, CD86, and CD40; expression of functional CC-chemokine receptor 7 (CCR7) and the maturation marker CD83; and heightened T-cell stimulatory capacity [reviewed in Ref. (15)]. The transcription factor E2-2 is essential for pDC development in both mice and humans (16). It controls the expression of pDC markers directly (e.g., TLR7, TLR9, BDCA2) and its deletion in mature pDCs, redirecting them toward the cDC phenotype. In contrast to cDCs, mouse pDCs are not phagocytic, and they maintain a high turnover of MHC class II, thus limiting their capacity as professional antigen-presenting cells (15).

More recently, a new DC subset defined as inflammatory DCs (infDCs) has been described in inflamed human tissues, including ascites of ovarian cancer (OC) and breast cancer (17). InfDCs ($CD14^+$ $CD16^-$ $BDCA1^+$) in cancer ascites were separated from macrophages ($CD14^+$ $CD16c^+$ $BDCA1^-$) and then further characterized for the expression of additional markers ($CD11c^+$ $CD11b^+$ $HLA\text{-}DR^+$ $BDCA1^+$ $CD206^+$). InfDCs were identified in inflamed tissue but not in tumor-draining lymph nodes with the exception of gastric cancer, which is known to be associated with persistent chronic inflammation. Molecular profiling of the purified infDCs revealed a close similarity with monocyte-derived DCs. These cells induce a Th17 differentiation *in vitro* and express two lineage-specific transcription factors, ZBTB46 and CSFR1, which were previously identified in mouse infDCs. Functional assays showed that infDCs could stimulate memory $CD4^+$ T cells from the same ascites to produce IL-17, likely by the secretion of IL-1β, IL-6, and IL-23, which are Th17 cell-polarizing cytokines (17).

Tumors can dramatically influence DC functions [reviewed in Ref. (6)]. It is well known that tumor-derived DCs are ineffective in stimulating an immune response and that this ineffectiveness may contribute to tumor evasion of immune recognition. Tumor-released factors can induce an altered myelopoiesis that leads to the release of immature myeloid cells, which, within the tumor bed, give rise to myeloid-derived suppressor cells (MDSCs). These findings, which have been confirmed in clinical studies, indicate a decreased presence and a defective functionality of mature DCs in patients with breast cancer (18), non-small cell lung cancer (19), pancreatic cancer (20), cervical cancer (21), hepatocellular carcinoma (22), and glioma (23). The fate of MDSCs has been investigated in various tumor types in relation to tumor drugs of different chemical nature including classical chemostatic agents, kinase inhibitors, and therapeutic antibodies. Pharmacological interventions, however, showed a marginal impact on DCs with respect to macrophages, which were skewed from an M2 (protumorigenic)

toward an M1 (anti-tumor) phenotype. Differentiation toward proinflammatory DCs was induced by vascular endothelial growth factor inhibitors (24) or blockers of chemokines (25).

How the immune system senses tumors is not as well defined as for non-self-antigen recognition. The danger theory proposes that detection of stressed or damaged cells by DCs is a driving force of adaptive immune responses, irrespective of the level of mutation frequency of a given tumor (26).

CANCER THERAPY, DC ACTIVATION, IMMUNE RESPONSES, AND DISCOVERY OF TUMOR ANTIGENS

Cancer is inevitably treated with different drugs that vary either in the mechanism of action (ranging from the original chemostatic alkylating agents to pathway-specific inhibitors) or in their chemical nature (small-molecule drugs, neutralizing antibodies, cancer vaccines, etc.). The ever-growing arsenal of context-specific anti-tumor drugs is likely to be applied in unpredicted tumor cases thanks to technical progress in global genome sequencing, for which low prices have made it almost an affordable diagnostic approach. The most utilized therapeutic approaches, however, remain those based on cytotoxic chemotherapy. Although it is well known that these drugs induce lymphopenia, it is becoming more and more appreciated that a subset of them also induces a series of DAMPs, which are recognized by PRRs on innate immune cells (**Figure 1**).

Damage-associated molecular patterns such as ATP and high mobility group box 1 protein (HMGB1) are secreted or released, whereas others such as calreticulin (CRT) and heat shock protein 90 are exposed *de novo* or become enriched on the outer leaflet of the plasma membrane (27) (**Figure 1**). In addition, DAMPs are produced as end-stage degradation products such as uric acid during the course of cell death. Most of these molecules have predominantly non-immunological functions inside the cell before their exposure on the cell surface or their secretion (28).

The group of Kroemer and Zitvogel (29) found that treatment with anthracycline in mice induces immunogenic cell death (ICD), which is mediated by CRT exposure on apoptotic cells (**Figure 1**). Researchers have also reported that timing of CRT exposure with respect to apoptotic markers and morphological changes is critical during ICD and that it usually anticipates apoptotic signs (29, 30). This is a structured process that occurs through different pathways including RNA-dependent protein kinase-like endoplasmic reticulum kinase (PERK)-mediated eIF2α phosphorylation, the secretory pathway, and caspase 8-mediated B-cell receptor-associated protein 31 (BCAP31)-dependent activation of BAX and BAK proteins (31).

An additional DAMP signal released during ICD is the HMGB1 (**Figure 1**). Preclinical studies have highlighted the importance of TLR4 activation mediated by HMGB1 binding. Research has shown that depletion of HMGB1 in mouse xenograft tumors prevents anthracycline-induced anti-tumor activity, which is restored by exogenous recombinant HMGB1 protein. Clinical studies in breast cancer have showed that a correlation exists between the presence of a single nucleotide polymorphism in the TLR4 gene, which prevents the binding of HMGB1 to TLR4, and early relapse after anthracycline treatment (32, 33). However, the role of HMGB1 can be context-specific, depending on the oxidation state: reduced HMGB1 performs as a chemoattractant DAMP, whereas the fully oxidized form is inactive (34, 35).

A recent paper by Ma and colleagues (36) has identified the cellular mediator of ICD to be specific inflammatory DC-like cells in mice. In particular, monocytes recruited into the tumor bed skewed toward a DC phenotype, which includes expression of inflammatory DC markers ($CD11c^+CD11b^+Ly6C^{hi}$). Tumors treated with mitoxantrone are infiltrated by $CD11c^+CD11b^+Ly6C^{hi}$ cells within 12 h of treatment and later by macrophages. This early infiltrate is responsible for the tumor-specific $CD8^+$ T-cell response and anti-tumor activity, as these effects are abrogated by local expression of ATP-degrading enzyme CD39, pharmacological blockage of purinergic receptors, and neutralizing antibody against CD11. The extracellular release of ATP is used not only in different pathways, such as survival, death, adhesion, proliferation, differentiation, and mobility, but also as a "find-me signal" from apoptotic cells, which attract monocytes expressing purinergic receptors (**Figure 1**). Release of ATP during the apoptotic process is mediated by autophagy, which is induced by some chemotherapy treatments. Research has shown that knockdown of essential autophagy genes (ATG5, ATG7, and BECN1) reduces ATP secretion in apoptotic cells treated with anthracycline and results in reduced anti-tumor activity *in vivo* (37, 38). Apparently, ATP affects DCs by acting on two pathways. ATP, at a concentration of about 1 μM binds and activates P_2Y_2 receptor that induces monocyte attraction. At concentrations higher than 30 μM, ATP binds to $P_{2\times7}$ receptor and activates NALP3-ASC-inflammasome, inducing secretion of IL-1β, which skews antigen presentation to $CD8^+$ T cells toward a Th-1 phenotype. The different activation threshold of $P_{2\times7}$ and P_2Y_2 receptors fits with a migratory/activation model, where low ATP concentrations in the periphery stimulate monocyte migration and higher ATP concentrations in the tumor bed induce DC differentiation.

The ICD concept presents new questions and challenges. Most of the mouse tumor models used to investigate ICD *in vivo* were based on tumor cell lines that did not evolve under an immunological pressure but rather were expanded *in vitro*. By contrast, the efficacy of immunogenic chemotherapy, such as the combination of oxaliplatin and doxorubicin, in spontaneous mouse tumor models has been shown to be independent of immune responses (39). Thus, it is important to determine the immunogenicity of anticancer therapies in humans and to identify which myeloid cells are recruited by chemotherapy.

Some evidence suggests that chemotherapy in humans is associated with antigen-specific immune responses (40, 41). Research on apoptotic antigens conducted previously in our laboratory has shown that caspase cleavage of self antigens derived from apoptotic cells facilitates their cross-presentation by DCs (42). Upon phagocytosis of apoptotic cells, the caspase-fragmented antigens can be efficiently exported by phagosomes into the cytosol, where they are processed through the class I-processing pathway and cross-presented in the form of peptides on class I molecules. In particular, self antigens, such as lamin B1, actin cytoplasmic 1, and vimentin, are normally sequestered in cell scaffolds; thus, they are unavailable for cross-presentation unless they are cleaved by caspases (43). The $CD8^+$ T-cell responses to these epitopes are present in chronic viral infections including those caused by

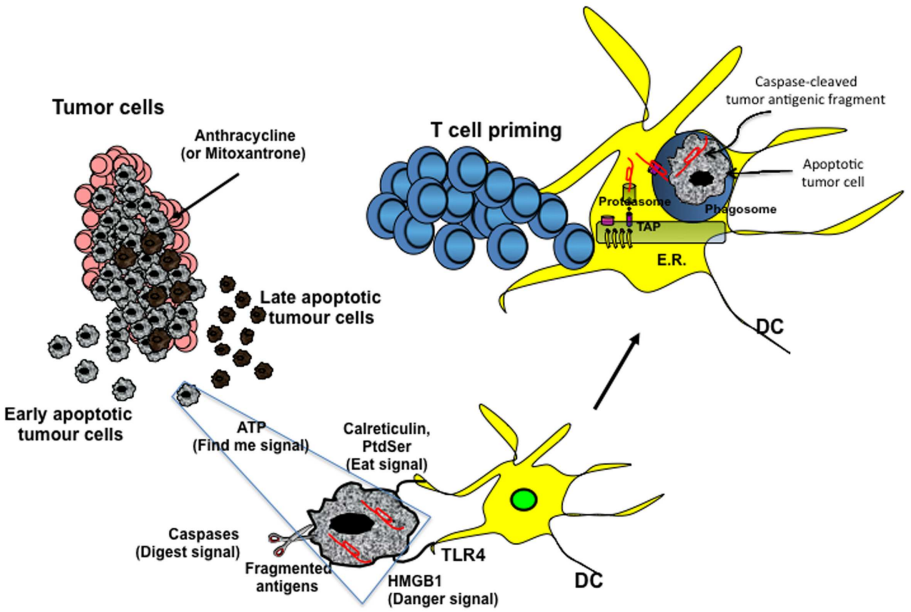

FIGURE 1 | Cross-presentation of chemotherapy-associated antigens derived from apoptotic tumor cells leading to anti-tumor immunity. Tumor cells, upon chemotherapy treatment (i.e., anthracycline), undergo immunogenic cell death. According to the Kroemer–Zitvogel model, the immunogenic death (apoptosis, necrosis, autophagy, etc.) of cancer cells involves a multistep process, including the release of "find-me" signals (such as fractalkine, nucleotides, and ATP) that attract phagocytes or dendritic cells (DCs), the expression of "eat-me" signals [such as phosphatidylserine (PtdSer) and calreticulin] that facilitate recognition by phagocytes or DCs, and, finally, the release of danger-associated molecular patterns [such as high mobility group box 1 protein (HMGB1) and other signals described in the text] that enable dying tumor cells to lose the propensity to induce tolerance and to stimulate powerful anticancer immune responses. An additional factor that is involved in the success of the immunogenic chemotherapy may emerge from the capacity of caspases to cut and release apoptotic cell-associated antigenic fragments (in red in the figure), thus facilitating their transport from phagosomes into the cytosol and the processing by DCs ("digest-me" signals) via the class I-processing pathways [the figure emphasizes the model suggesting that caspase-cleaved apoptotic fragments are trimmed by cytosolic proteasomes in the form of peptides and that TAPs transport the resulting apoptotic self epitopes into the lumen of the endoplasmic reticulum (ER), where they can bind the appropiate class I molecule]. The final goal of these multiple checkpoints is to cross-present tumor epitopes and to elicit a wide repertoire of memory tumor-specific $CD8^+$ T cells in patients undergoing tumor regression in response to appropriate chemotherapeutic regimes. These tumor-specific T cells represent a principal tool for discovering immunogenic tumor antigens by interrogating those responding to highly purified tumor proteins.

human immunodeficiency virus I and hepatitis C virus (42, 44), as well as in multiple sclerosis patients (45), and correlate with the disease progression.

To verify whether chemotherapy-induced apoptosis is immunogenic in humans, we analyzed OC patients who were treated with chemotherapy in the adjuvant setting (46) (**Figure 1**). To identify the immunogenic chemotherapy-associated antigens (CAAs), memory T cells from OC patients were interrogated with proteins isolated from primary OC cells by evaluating their response to two-dimensional electrophoresis gel-eluted OC proteins. Immunogenic CAAs were then molecularly characterized by mass spectrometry (MS)-based analysis. Memory T-cell responses against CAAs derived from apoptotic (but not live) OC cells correlated with prolonged survival in response to chemotherapy, thereby supporting the model of chemotherapy-induced apoptosis as an adjuvant of anti-tumor immunity (46). In addition, memory $CD8^+$ T cells specific for individual OC proteins were elicited upon cross-presentation of CAAs or whole apoptotic (but not live) OC cells, suggesting that cross-presentation of tumor antigens and T-cell responses could contribute to the efficacy of anticancer chemotherapy. The antigen-specific $CD4^+$ and $CD8^+$ T-cell responses that were originally observed in the screening with proteins extracted from primary cancer cells were further confirmed using corresponding recombinant proteins. It is interesting to note that antigen-specific $CD4^+$ and $CD8^+$ T cells produced either IFN-γ or IL-17, which is in line with the recently described Th17 cell-polarizing infDCs described in human OC cases (17). MS-based analysis of CAAs showed enrichment for proteins of stress pathways such as Ras-related protein, heat shock protein β1, and heat shock protein α-B-crystallin. Taken together, these data suggest that CAAs correspond not necessarily to tumor cell-specific antigens but rather to ubiquitous proteins, which, under normal conditions, are sequestered in cell structures that limit their processing and presentation to T cells. However, as a result of the chemotherapy effects, apoptosis of tumor cells can induce upregulation of a wide range of ubiquitous proteins sufficient for subsequent processing and presentation by DCs, which in turn could prime the corresponding specific T cells (**Figure 1**).

There are many open questions surrounding how DCs can drive immune responses during chemotherapy in humans and whether the memory immune response against CAAs plays any role in preventing tumor relapses. We believe that the identification of new immune correlates can help in refining a more targeted and effective anticancer therapy.

AUTHOR CONTRIBUTIONS

Fabio Palombo conducted the literature review and cowrote the manuscript, Chiara Focaccetti helped in finalizing the manuscript, and Vincenzo Barnaba provided overall supervision and cowrote the manuscript.

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The dark side of dendritic cells: development and exploitation of tolerogenic activity that favor tumor outgrowth and immune escape

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Dendritic cells (DC) play a central role in the regulation of the immune responses by providing the information needed to decide between tolerance, ignorance, or active responses. For this reason different therapies aim at manipulating DC to obtain the desired response, such as enhanced cell-mediated toxicity against tumor and infected cells or the induction of tolerance in autoimmunity and transplantation. In the last decade studies performed in these settings have started to identify (some) molecules/factors involved in the acquisition of a tolerogenic DC phenotype as well as the underlying mechanisms of their regulatory function on different immune cell populations.

Keywords: dendritic cell, tumor, immune escape, tolerance, immunotherapy

INTRODUCTION

The immune system evolved with the difficult task of preserving the integrity of the "self," while protecting it from "non-self" and/or dangerous invaders, thus finding the right balance between aggression and tolerance. A central role in orchestrating the different immune cell subpopulations is played by dendritic cells (DC), the major professional antigen presenting cells (APC). Over the last two decades, many different DC subsets have been identified and classified into myeloid DC, which comprise all monocyte-derived cells and blood-resident $CD1c^+$ DC and into plasmacytoid DC (PDC).

A particularly difficult task for the immune system is to fight tumors, since they derive from the "self," but based on their high proliferative potential they are dangerous for the survival of the host. Moreover, due to the high mutation rates of tumor cells the selection pressure posed by an immune response can result in tumor immunoediting with the outgrowth of immune escape variants or the induction of a suppressive microenvironment. In line with the central role of DC in balancing response versus tolerance, many of the immune escape mechanisms displayed by cancer cells affect DC. These include alterations in the frequency and/or function of circulating and tumor-infiltrating DC in patients with tumors of different histologies. In particular, DC in cancer patients can be affected in their differentiation capacity, with either enhanced apoptosis or skewed phenotype toward immature cells with suppressive properties collectively named myeloid derived suppressor cells [MDSC; (1, 2)], in their ability to process and/or present tumor-associated antigens (TAA) and in their ability to interact with effector cells, e.g., to activate and/or correctly polarize them.

Studies performed with DC differentiated *in vitro* in the presence of tumor cells or of their conditioned medium as well as with purified tumor-infiltrating DC identified the underlying mechanisms responsible for such alterations leading to a pro-tumorigenic phenotype. This review summarizes the known processes employed by tumor cells to subvert professional APC (summarized in **Table 1**) and how the increasing knowledge can not only help in fighting cancer, but also in inducing tolerance to transplanted organs and suppression of autoimmune diseases.

MYELOID DC AND CANCER

Tumor cells can influence the phenotype and function of myeloid cells at different time points of their life and with distinct mechanisms. These include the metabolic shift of tumor cells toward the anaerobic glycolytic pathway for glucose degradation resulting in increased concentrations of extracellular lactate and an acidification of the microenvironment, the so-called Warburg effect (3). Monocytes cultured *in vitro* in the presence of lactate and low pH have shown an impaired differentiation toward DC favoring either an expansion of MDSC (4) or of macrophages that promote a Th17 polarization (5). Despite prolonged incubation in the presence of lactate impairs DC responsiveness to lipopolysaccharide [LPS; (6)], a transient exposition promotes DC maturation and enhances their ability to induce a type 1 immune response (7). In addition to pH alterations, the tumor microenvironment is characterized by hypoxia that skews DC toward a type 2 polarization (8), reduces their ability to uptake antigens (Ag), and alters their migratory properties (9).

In addition, expression of hyaluronan (HA), a component of the extracellular matrix of the tumor stroma, correlates with tumor invasiveness and poor survival of patients with ovarian, breast, and colorectal cancer (10–13), while high HA levels correlate with more differentiated tumor phenotype and an enhanced survival in patients with oral squamous carcinoma (14). The effects of HA

Table 1 | Effects of tumor-derived molecules on APC functions.

APC properties	Factor	Effects[a]	Reference
Differentiation	Ganglioside	Reduced CD1a. Reduced DC from CD34 progenitor	(77, 78)
	HA	Suppressive Mf promoted over DC	(18)
	HLA-G	Promoted expansion of MDSC *in vivo*	(27)
	Lactate/pH	Impaired differentiation (no CD1a), promoted MDSC expansion	(4, 6)
	Mucins	More immature phenotype	(59, 60)
	PGE_2	Promoted MDSC differentiation	(69)
	VEGF	Promoted MDSC differentiation	(40–42)
	Wnt5a	Impaired differentiation of monocytes toward mDC	(53, 55)
Migration	Ganglioside	Lower CCR7 and impaired migration toward CCL19 (LC) and CCL3	(74, 78)
	Hypoxia	Enhanced migration toward SDF-1α and CCL4; reduced CCR7 levels	(8, 9)
	PGE_2	Enhanced expression and functionality of CCR7 (mDC). Reduced CCR7/CXCR4 ratio for tissue retention (PDC)	(62, 100)
	TGF-β	Reduced migration *in vivo* to LN and *in vitro* to CCL19; enhanced expression of inflammatory CCR	(44, 45)
Ag uptake and processing	Ganglioside	Reduced expression of various APM components; reduced endocytosis	(78, 79)
	HLA-G	Reduced MHC class II antigen processing	(22)
	Hypoxia	Reduced endocytosis	(8, 9)
	TGF-β	Reduced endocytosis and phagocytosis	(44)
	Wnt5a	Lower fluid phase and CD206-mediated Ag internalization	(55)
Surface molecules	Ganglioside	Lower CD40, CD54, CD80, CD86, CD83 (LC, mDC)	(74, 77, 82)
	Glycodelin	Reduced CD83 and CD86	(33)
	HLA-G	Reduced HLA-DR, CD80 and CD86	(22, 24–26)
	Hypoxia	Reduced CD40 and HLA-DR	(8)
	IL-10	Reduced CD86	(177)
	Mucin	Reduced CD40, CD83 and CD86	(58–60)
	PGE_2	Enhanced OX40L and CD70 induction (mDC). Reduced CD40 (PDC)	(63, 64, 100)
	TGF-β	Reduced CD80 and CD40	(44)
	Wnt5a	Reduced CD80 and CD86 (PDC)	(56)
Secreted molecules	Ganglioside	Reduced IL-6, IL-12 and TNF-α, increased PGE_2 secretion	(59, 82)
	Glycodelin	Enhanced IL-6 by monocytes and Mf. Reduced IL-12 and higher IL-10 in mDC	(33, 34)
	HA	Enhanced IL-10 by suppressive Mf. Reduced IL-12/IL-10 ratio in mDC	(18, 19)
	HLA-G	Reduced IL-12, enhanced IL-6	(24, 26)
	Hypoxia	Reduced IL-12 and TNF-α and enhanced IL-10	(8)
	IL-10	Reduced IL-12 and/or IFN-α production (PDC)	(101, 102)
	Lactate/pH	Reduced IL-12, IL-6 and TNF-α; enhanced IL23	(5, 6)
	Mucin	Reduced IL-12, increased IL-10	(58–60)
	PGE_2	Reduced IL-12/IL23 ratio, reduced CXCL10, CCL5 and CCL19; enhanced IDO (mDC). Reduced IFN-α and TNF-α (PDC)	(66, 70, 71, 100, 102)
	sCD83	Enhanced TGF-β and consequently IDO (PDC, mDC)	(51)
	TGF-β	Reduced IFN-α and TNF-α (PDC)	(100, 103)
	Wnt5a	Inhibited IFN-α secretion (PDC); enhanced TGF-β and IL-10; reduced IL6 and IL-12	(54–56)
Survival	Ganglioside	Enhanced apoptosis (LC, mDC)	(74, 75)
	Glycodelin	Contradictory results	(34)
	HA	Enhanced apoptosis via NO induction	(20)
	IL-10	Enhanced apoptosis (PDC)	(101)
	Mucins	Enhanced apoptosis early during differentiation	(57)
Interaction with NK cells	HLA-G	Reduced activation (CD69, IFN-γ secretion, cytotoxicity)	(26)
	PGE_2	Reduced recruitment and induction of IFN-γ	(71)

(Continued)

Table 1 | Continued

APC properties	Factor	Effects[a]	Reference
Interaction with nk-T cells	TGF-β	Reduced CD1d and lipid presentation	(46)
Interaction with T cells	Ganglioside	Reduced allo-MLR (LC). Reduced proliferation to TT and allo-MLR (mDC)	(74, 78, 81, 82)
	Glycodelin	Reduced induction of proliferation. Reduced IFN-γ secretion	(28, 34)
	HA	Enhanced T cell apoptosis via ROS production	(19)
	HLA-G	Reduced allo-MLR, more IL-10 secreting $CD8^+$ T, anergic $CD4^+$ T	(22, 25)
	Hypoxia	Enhanced IL-4 over IFN-γ secretion, type 2 skew	(8)
	IL-10	Enhanced proliferation of $CD4^+$ T and skew toward Th2 (PDC). Reduced allo-MLR and anergy induction	(102, 177)
	Lactate/pH	Reduced Ag specific $CD8^+$ T proliferation; enhanced IL-17 over IFN-γ secretion	(5, 6)
	Mucin	Reduced allo-MLR, reduced IFN-γ secretion by $CD8^+$ T	(60)
	PGE_2	Enhanced IL-17 and reduced IFN-γ, inhibition via IDO and soluble CD25 (mDC). Enhanced proliferation of $CD4^+$ T and skew toward Th2 (PDC)	(65, 66, 70, 102)
	sCD83	Induction/expansion of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg	(51)
	TGF-β	Reduced proliferation in allo-MLR and to peptide, reduced IFN-γ secretion	(44)
	Wnt5a	Reduced IFN-γ secretion, higher IL-10 secretion. Reduced proliferation	(54, 55)

[a]*When nothing given between brackets, mDC are considered.*

PDC, plasmacytoid DC; LC, Langerhans cells; Mf, macrophages; mDC, myeloid DC; LN, lymph node; MLR, mixed leukocyte reaction.

on DC are controversial and possibly related to its size: whereas low molecular weight HA can induce DC maturation *in vitro* (15, 16) and improve their functionality *in vivo* as cancer vaccine (17), intermediate sized HA impairs monocyte differentiation resulting in immunosuppressive APC characterized by a macrophage-like phenotype ($CD14^+$, $CD1a^{low}$), a reduced upregulation of costimulatory molecules and inflammatory cytokines after stimulation with toll-like receptor (TLR) ligands and an enhanced secretion of interleukin (IL)-10 (18, 19). Moreover, HA-conditioned DC can secrete nitric oxide (NO) and reactive oxygen species (ROS) that can induce apoptosis in DC and in co-cultured T cells, respectively (19, 20).

An other escape strategy exploited by tumor cells is the hijacking of endogenous mechanisms of tolerance induction used by immuno-privileged organs. This is mediated by the non-classical HLA-G antigen, which exhibit a tightly controlled physiologic expression restricted to cornea, thymic epithelial cells, reproductive organs, embryonal tissues, and the extravillous cytotrophoblasts at the maternal-fetal interface. Furthermore, HLA-G is often expressed in solid and hematologic tumors either as a transmembrane and/or a secreted/shed protein, thereby protecting tumor cells from the cytolytic activity of natural killer (NK) and T cells (21). In addition, HLA-G can also impair myeloid DC by binding to the inhibitory receptors ILT2 and ILT4 in humans and PIR-B in mice (22–24). Receptor triggering by HLA-G inhibits the nuclear translocation of the transcription factor NF-κB (25), which is consequently accompanied by reduced expression of costimulatory molecules and proinflammatory cytokines as well as impaired presentation of MHC class II-restricted epitopes (22). As a consequence, HLA-G treated DC lack the ability to induce NK cells activation (26) and promote anergy of effector cells and differentiation of regulatory T cell [Treg; (22)]. Furthermore, tumor-expressed HLA-G induced suppressive MDSC and tumor growth *in vivo* (27).

Glycodelin (previously called placental protein 14 or PP14, α2-globulin, progesterone-associated endometrial protein or zona-binding inhibitory factor) has been originally identified as the molecule responsible for the immunosuppressive activity in the decidua during early gestation (28), but is also expressed in tumors of the reproductive tract, e.g., ovarian carcinoma, where its glycosylated form glycodelin A (GdA) correlated with unfavorable prognosis (29). Furthermore, glycodelin correlate with a worse patients' prognosis in familiar, non-BRCA1/2 breast carcinoma (30) and in lung cancer (31). *In vitro* characterization of glycodelin function demonstrated suppressive effects on all immune cell populations (32), including DC. Treatment of DC with GdA results in lower expression levels of costimulatory molecules, a low IL-12/IL-10 ratio (33), and a reduced ability to induce a type 1 polarization of effector cells (34). Depending on the culture conditions, GdA has also been reported to induce or suppress apoptosis in monocytes [see discussion in Ref. (34)].

Other "physiologic" tolerogenic factors borrowed by tumor cells include indoleamine 2,3-dioxygenase [IDO; (35)], adenosine production via CD73 expression (36), and secretion of IL-10 (37, 38), transforming growth factor-β [TGF-β; (39)] or soluble CD83 (sCD83).

Transforming growth factor-β plays not only a role in MDSC development, like vascular endothelial growth factor [VEGF; (40–42)], IL-6 and/or macrophage colony stimulating factor [M-CSF; (43)], but also impairs the DC migratory capacity by altering the expression pattern of chemokine receptors (44, 45) and inducing downregulation of CD1d thus impairing DC interactions with NK-T cells (46).

sCD83 was found in total blood cell cultures after stimulation and might represent a feed back mechanism to shut down an immune response (47). Indeed, enhanced serum levels of sCD83 detected in hematologic malignancies and solid tumors, like lung carcinoma correlate with shorter tumor-free survival (48–50).

In vitro treatment of DC with recombinant sCD83 results in enhanced IDO production and induction of TGF-β producing Foxp3$^+$ Treg (51).

An additional strategy of immune escape mechanisms exploited by tumor cells consists in the upregulation of molecules with negative effects on DC. These include alterations in the Wnt/β-catenin pathway inducing activation of MerTK receptor (c-mer proto-oncogene tyrosine kinase) in infiltrating cells like macrophages and DC that help tumor growth *in vivo* (52). *In vitro* studies have found that tumor-derived Wnt5a can impair the differentiation of monocytes toward DC (53) and inhibit the maturation response to TLR ligand by myeloid DC (54, 55) as well as by PDC (56).

Mucins are expressed by many epithelial tumors and their presence during differentiation of monocytes toward DC results in less differentiated cells with increased apoptosis (57), impaired response to TLR ligand stimulation, cytokine production skewed toward the immunosuppressive IL-10, impaired ability to induce proliferation of T cells, and enhanced induction of suppressive T cells (58–60). Those effects seem to be mediated by binding to the mannose receptor, siglec-3 and -9 (57–59).

A hallmark of many tumors is the secretion of high levels of prostaglandin E_2 (PGE_2) due to upregulation of cyclooxygenase (COX)1/2. The consequences of PGE_2 on DC functionality are complex. While it represent a component of the "gold standard" cocktail for vaccine DC maturation (61) due to its role in promoting CCR7-mediated migration (62), and it also induces the expression of costimulatory molecules like OX40-L and CD70 promoting T cell functions (63, 64), it can inhibit the synthesis of IL-12p70 (65), while favoring the secretion of IL-23 that promote Th17 immune responses (66) and tumor development (67, 68). Moreover, PGE_2 enhances MDSC differentiation (69), induces expression of IDO and soluble CD25 that inhibit T cell stimulation (70) and impairs the cross talk with NK cells (71). A possible explanation for the contrasting effects can be due to the specific receptor triggered by PGE_2 (72) and/or the relative ratio between PGE_2-treated DC and effector cells (73).

Altered and/or secreted gangliosides have also been demonstrated to affect DC differentiation and survival (74–78). Moreover, gangliosides impair the ability of monocytes to induce T cell proliferation due to a downregulated expression of components of the antigen processing machinery [APM; (79, 80)], a suppressed costimulation and a reduced cytokine production (81, 82). *In vivo*, a correlation between elevated levels of the ganglioside GM3 and a higher frequency of immature DC was found in non-small-cell lung cancer (83).

Furthermore, "tumor-deviated" DC/MDSC exhibit an altered phosphorylation pattern of STAT3 (84, 85) that has also been linked to the inhibition of IL-12p40 transcription (86) and/or of p38 (87) that is involved in the induction of Th17 responses (88).

In addition to boost the immune suppression, tumor-conditioned DC can also provide direct help to tumor cells by secreting mitogens for the tumor cells (89), by favoring the epithelial mesenchymal transition (90), by promoting their invasiveness and ability to metastasize (8, 90) and by inducing angiogenesis (91, 92).

PDC AND CANCER

Plasmacytoid DC have been found in the infiltrate of various human solid tumors like melanoma, breast, ovarian, and head and neck carcinoma, where they frequently correlated with a worse patients' prognosis (93–96). Functionally, PDC can be recruited by the tumor through its secretion of CXCL12 (also called SDF-1α) and CCL20 (97–99). Then, factors locally released by tumor cells, like TGF-β, tumor necrosis factor (TNF)-α, IL-10, and PEG_2 (95, 100–104) as well as triggering of the PDC-specific receptor ILT7 (105, 106) induce the immunosuppressive properties of PDC. Indeed, tumor-conditioned PDC display a semi-mature phenotype with expression of costimulatory molecules but impaired secretion of IFN-α (93, 101, 103). In addition, tumor-associated, tolerogenic PDC showed an upregulation of the transcription factor Foxo3 (107) and an impaired migration to lymphoid organs due to reduced CCR7 expression (100).

Characterization of the immunosuppressive activity of PDC *in vitro* have highlighted their ability to induce unresponsiveness of effector cells, to promote the development of suppressive CD8$^+$ T cells, to differentiate naïve CD4$^+$ T cells toward Foxp3$^+$ or IL-10 producing Treg as well as to expand pre-existing Treg (108–113). From the molecular point of view, important roles have been identified for ICOS ligand (ICOS-L), IDO, notch ligand delta-like 4 (Dll4), and granzyme B. ICOS-L is upregulated shortly after maturation induced by CD40-L or TLR9 triggering (108), is involved in inducing IL-10 production in CD45RO$^+$ T cells (114) and in sustaining the survival and proliferation of Foxp3$^+$ Treg (115). A role *in vivo* for this pathway is supported by the co-localization between ICOS$^+$ Treg and ICOS-L$^+$ PDC within breast and ovarian carcinoma (115, 116). Murine and human PDC can produce IDO *in vitro* upon triggering of TLR9, CTLA-4, GITR, or CD200 (117–119). PDC expressing IDO have been identified in melanoma draining lymph nodes in murine models and human patients and have been correlated with the activation of naïve and mature Treg (120–122). In murine models, the constitutively expressed Dll4 allow PDC to induce Th1 cells to produce IL-10 even under type 1 polarizing conditions, thus favoring the shut down of an immune response (123). Granzyme B, whose secretion by PDC is boosted by tumor-derived IL-3 and IL-10, is involved in the downregulation of the CD3ζ chain of T effector cells, thereby resulting in their anergy or deletion by apoptosis induction (124, 125).

In addition to their immunosuppressive role, PDC play a protumorigenic role by promoting angiogenesis via secretion of TNF-α and IL-8 (126) and favoring metastasis dissemination into the bone (127).

IMPROVED PROTOCOLS FOR DC-BASED VACCINATION AGAINST CANCER

Two major strategies of DC-based tumor immunotherapy have been implemented. The first is based on the *ex vivo* production and manipulation of DC that are then injected into the patients while the second targets the DC directly *in vivo* (**Figure 1**).

The classical strategy for the first approach consists in the differentiation of CD14$^+$ circulating monocytes or CD34$^+$-mobilized precursor cells into immature DC by culturing them in the presence of granulocyte-monocyte colony stimulating factor (GM-CSF) and IL-4 for 7 days, after which they are loaded with TAA

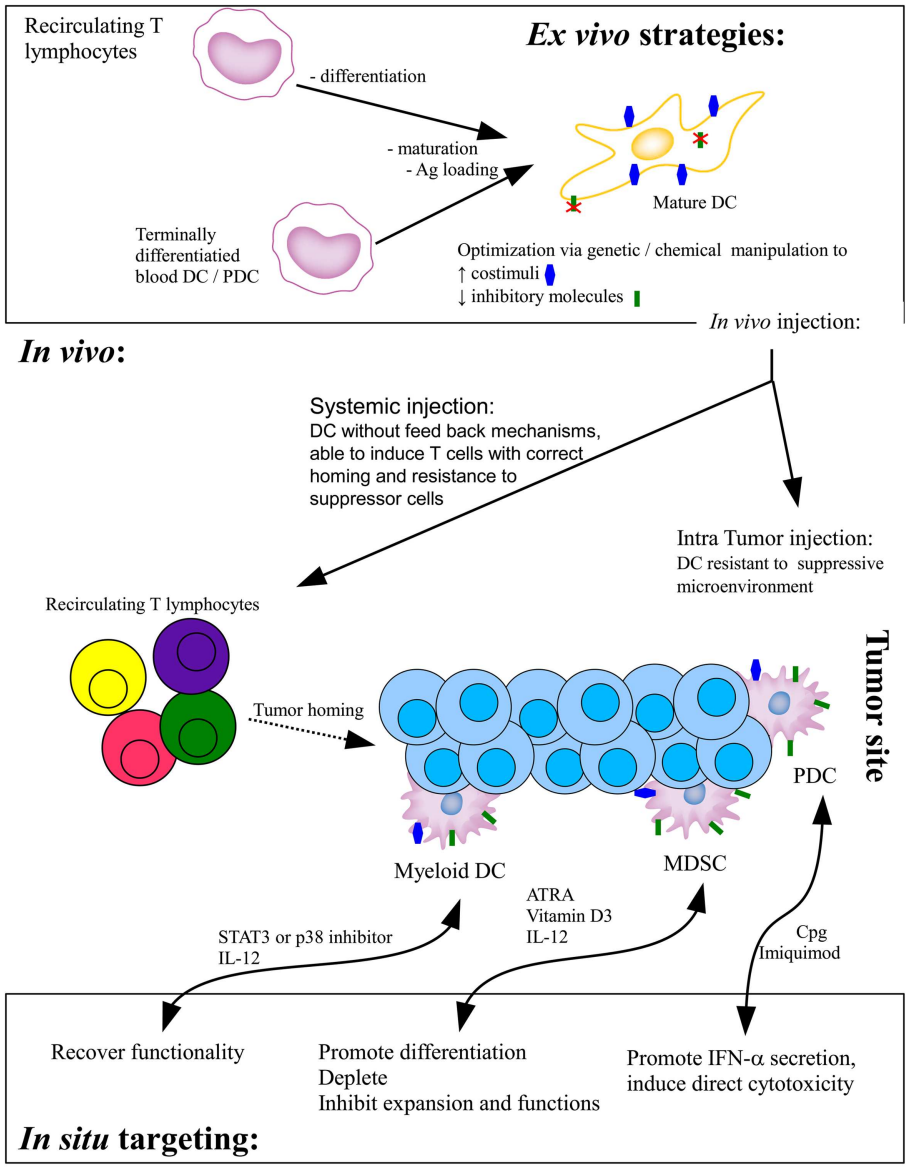

FIGURE 1 | Current DC-based strategies of tumor immunotherapy. In the *ex vivo* strategy, monocytes-derived immature DC or terminally differentiated blood DC are loaded with tumor antigens and/or induced to mature before *in vivo* injections. Whereas systemically injected DC will migrate to the draining lymph node to prime effector T cells, intratumorally injected DC have to interact with effector cells within the suppressed microenvironment. Direct *in situ* targeting strategies aim at recovering the functionality of infiltrating DC, either promoting their correct differentiation or providing stimuli to foster their functionality. Ag, antigen; ATRA, all-trans retinoic acid.

and induced to mature before *in vivo* injection. Studies performed using cells from patients with different solid as well as hematologic cancer histotypes have demonstrated that precursor cells are either not irreversibly impaired and can be matured with this protocol or that is possible to rescue their differentiation into functional DC upon inhibition of STAT3, p38, and/or IL-6 (128, 129). Despite the good results obtained *in vitro* with patient-derived DC, and the induction of immune response in treated patients demonstrated by expanded Ag-specific T cells and delayed type hypersensitivity (DTH) reactions, the first clinical trials with vaccine DC resulted in poor clinical outcome. Based on the increased knowledge of the DC biology and of tumor escape mechanisms the protocol for the *ex vivo* production of vaccine DC has to be optimized (**Box 1**).

The initial poor results of DC-based immunotherapy could be due to the immature or only partially mature phenotype of the DC, and in particular to their reduced levels of IL-12 secretion. Thus, many alternative maturation protocols have been developed, which induce DC with an enhanced IL-12 secretion and functionality *in vitro*, with some of them that have also reached clinical application. The "alpha type-1 polarized DC" obtained upon maturation in the presence of IL-1β, IFN-α, IFN-γ, poly IC, and TNF-α (130) have been tested in patients with recurrent glioma

Box 1 | Optimization of DC-based tumor immunotherapy.

Different anatomical and tumor derived factors pose problems to the success of DC-based therapy. Following is a list of key points that have to be optimized.

(A) *Ex vivo* DC preparation
- DC subset: terminally differentiated blood DC (PDC, $CD1c^+$ DC, mixed) or monocyte derived DC (GM-CSF + IL-4, GM-CSF + IFN-α, GM-CSF + IL-15; standard 7 days or shortened 2–3 days protocol).
- Antigen loading: protein, DNA, or mRNA; one or multiple Ag, defined or total tumor repertoire.
- Maturation: TLR-ligand(s) (poly IC, MPLA, R848) and/or immune-derived (CD40-L, IFN-γ). Is PGE2 to be added for the migratory ability?
- Targeted effector cells: $CD8^+$ T cell only; also $CD4^+$ T helper and/or innate effector cells (NK, iNKT, γδ T cells)

(B) Vaccination protocol:
- Injection route: intratumor versus systemic (intradermal, intramuscular, subcutaneous, intranodal)
- Number of injection and distance in between
- Optimal DC dose
- Combination with other treatment modalities (remove suppressive populations, reduce local immunosuppression, enhance tumor permeability. . .)

(131, 132), melanoma, and colorectal cancer (NCT00390338 and NCT00558051 at http://www.clinicaltrials.gov, respectively), whereas DC stimulated with LPS and IFN-γ have been used for the treatment of patients with breast cancer (133, 134). DC stimulated with the streptococcus-derived immunotherapeutic agent OK432 have been used against hepatocellular carcinoma (135) and colorectal cancer (136).

In parallel to the manipulation of the maturation protocol, the type of DC was also optimized. Alternative differentiation protocols for monocytes have been tested to obtain more physiologic DC types. GM-CSF has been combined with IFN-α to induce inflammatory DC, which have already been tested in patients with medullary thyroid carcinoma (137), or with IL-15 to induce Langerhans-like DC that despite the enhanced functionality *in vitro* did not provide higher responses in melanoma patients when compared to standard DC (138). Furthermore, terminally differentiated DC have been used both as single or mixed populations. Regarding the myeloid subset, in a preclinical trial sufficient amount of $CD1c^+$ blood DC have been purified from healthy as well as melanoma and Bowel cancer disease patients under GMP (good manufacturing practice) conditions and could be induced to secrete proinflammatory cytokines, thus opening the way for a possible clinical application (139). Two different approaches using PDC have been developed. A leukemic cell line with PDC characteristic has been isolated and, after having demonstrated functional activity in humanized murine models (140) and with melanoma PBL *in vitro* (141) will be evaluated in a clinical trial in HLA-matched melanoma patients (NCT01863108). In contrast, de Vries and co-workers have employed autologous, patient-derived PDC in a phase I clinical trial against melanoma (142). A GMP platform has been established to purify all subtypes of circulating APC resulting in a population able to induce Ag-specific CTL both from healthy donors and myeloma patients (143). The injection of a highly purified DC population does not seem to be required since the sipuleucel-T (also called APC8015 or Provenge®) vaccination approved by the Food and Drug Administration (FDA) for treatment of prostate cancer patients is based on a highly mixed population, in which the DC targeted with TAA only represent a small component (144, 145).

Other optimizations have been also evaluated in order to modulate the suppressive environment that impair the *in vivo* ability of the vaccine DC to prime immune responses. This is mediated by rendering DC insensitive to the tumor-induced suppressive microenvironment by blocking inhibitory signaling pathways, like TGF-β (146, 147), IL-6 (148), and STAT3 (129, 149, 150). On the other side, the costimulatory function of DC have been further improved by providing the T lymphocytes with all required positive signals and/or the absence of negative feedback regulators in order to acquire full functionality and resistance to suppressor cells. DC unable to produce IL-10 (151, 152), insensitive to CTLA-4 triggering (153), providing enhanced levels of CD70 (154–157), CD80 (154), or GITR-L costimulation (153, 158) have proved to induce T cells with enhanced resistance to Treg suppression, delayed induction of tolerance as well as reversion of the tolerized status. Some of those "costimulatory enhanced" DC have also started the path of clinical trials like the TriMix DC (expressing CD40L, CD70, and a constitutively active TLR4 receptor) in melanoma patients (159, 160).

To provide a more general pro-stimulatory phenotype, multiple signaling pathways have also been enhanced by either inducing expression of the transcription factor T-bet (161, 162) or by silencing A20, an inhibitor of signaling pathway downstream of TLR and TNF receptors (163) resulting in DC with improved functionality. Similarly, with the increased knowledge of the important role of micro RNA (miR) in the fine tuning of gene transcription and their role in the immune response and in DC functions (164), DC-specific miR have been targeted. For example, inhibition of miR-22 and miR-503, two miRNA upregulated in DC upon coculture with tumor cells, resulted in improved therapeutic activity due to enhanced DC survival (165).

The major aim of the second line of therapy is to revert the tolerized phenotype of tumor-infiltrating and/or recirculating DC in order to allow proper activation of effector cells. The most clinically advanced strategies are those focusing on PDC and using ligands of the TLR-7 and -9 to recover their IFN-α secreting capabilities. After the successful use of imiquimod and CpG-containing oligonucleotides in murine models, many clinical trials have also been performed (166), leading to the approval of imiquimod for

cancer immunotherapy by the European Medicines Agency and the FDA. A problem with such a strategy is the fact that in some, but not all tumors a downregulation of the two TLR in tumor-infiltrating PDC have been reported (93). At the basis of the positive outcome upon PDC *in situ* triggering there can be not only the activation of other immune cells and the inhibition of Treg (167), but also a direct tumoricidal activity upon upregulation of TRAIL and granzyme B (168, 169). Is to be underlined that the upregulation of granzyme B by PDC can also have detrimental effect by killing T cells (124, 125, 169). An other reported effect of CpG injection is the differentiation of MDSC toward functional monocytes with consequent reduction in the amount of suppressive cells (170, 171).

On the side of targeting myeloid DC *in vivo*, similar approaches to the *ex vivo* manipulation have been tested. Chemical inhibitors of negative signaling pathways like STAT3 (150), or positive modulators like miR-155 (172), have been injected *in vivo* with the aim to be uptaken by DC and to revert their tolerogenic phenotype and promote immune-mediated tumor rejection. Provision of missing IL-12 through different technical approaches have demonstrated that in addition to the stimulation of NK and T cells, also myeloid cells are positively affected with the activation of cytotoxic macrophages and reversion of MDSC with loss of their suppressive properties (173, 174). Many different strategies are also aiming at the removal of MDSC acting on their differentiation and/or suppressive functions (175). For example all-trans retinoic acid (ATRA), a compound reducing MDSC number and function has been combined to DC vaccination in a phase I trial in small cell lung cancer patients (176).

EXPLOITATION OF TOLEROGENIC DC IN TRANSPLANTATION AND AUTOIMMUNITY

In the setting of autoimmunity and transplantation the aim of immunotherapy is to reduce inflammation and to induce a local and/or antigen-specific immunosuppression/tolerance in order to avoid organ rejection and reduce the disease score without increasing the risk of opportunistic infections. Like for tumor immunotherapy, DC have been manipulated *ex vivo* or directly targeted *in situ*.

Tolerogenic DC have been differentiated *in vitro* from human monocytes and murine bone marrow cells upon culture in the presence of different combination of IL-10 (177), TGF-β (178), vitamin D3 (179), dexamethasone (180), protein kinase C inhibitor (181), and rapamycin (182). These cells are characterized by a semi-mature phenotype, the ability to expand Treg and to preserve such properties even in an inflamed microenvironment, as mimicked by stimulation with TLR ligands (183, 184). Murine models of organ transplantation as well as different autoimmune diseases have demonstrated the therapeutic applicability of such tolerogenic DC (185–188) and opened the way to preclinical evaluation in multiple sclerosis (189) as well as in a phase I trial in rheumatoid arthritis [RA; (190)]. Similarly, the good results obtained with non-obese diabetic (NOD) mice injected with DC silenced in the major costimulatory molecules [i.e., CD40, CD80 and CD86; (191)] have opened the way for a phase I safety study in patients with type 1 diabetes (192). In the setting of organ transplantation, "classical" murine bone marrow-derived DC from the organ donor have been either triggered with tetramer of sHLA-G1 (25) or silenced in NF-κB (193) in order to induce a transplant-specific tolerance that allow (longer) acceptance of the graft.

Direct *in situ* targeting of DC has also been implemented in order to promote local and/or antigen specific tolerance. Clinical trials have been performed with apilimod (or STA5326), a specific inhibitor of IL-12 and IL-23, which are the central mediators of the Th1 and Th17 responses involved in autoimmunity. Whereas in psoriasis a reduction in inflammatory cytokine and DC infiltration of the skin lesions was observed upon apilimod (194), no robust clinical improvement was found in RA (195) and contrasting results were reported in Crohn's disease (196, 197). Although still in the preclinical phase systemic or topic injection of sCD83 was able to prolong survival of grafted organs (51, 198) as well as to reduce experimental autoimmune encephalomyelitis (EAE) in both prophylactic and therapeutic setting (199). Additional strategies inducing antigen specific tolerance consist in coupling the desired antigen to antibodies or ligands for specific receptors expressed by DC. Examples are DEC-205 (200, 201), the human DC immunoreceptor (DCIR) (202, 203), and the murine acid binding Ig-like lectin H [siglec-H; (204)] that induce specific tolerance to the antigen they have been targeted with.

CONCLUSION

Despite the ever growing knowledge on the immunologic function of DC and how tumor cells try to subvert them, a long way has still to be performed before defining the best protocol of vaccination regarding not only the maturation/resistance of the DC but also the road of injection, the number of injections, the type of antigen(s) and the loading strategy (see **Box 1**). Of particular interest is the recent report that in therapeutic setting a single immunization performs better alone that with a following boost, a setting that is on the contrary highly favorable in prophylactic immunization (205).

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Pathological mobilization and activities of dendritic cells in tumor-bearing hosts: challenges and opportunities for immunotherapy of cancer

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A common characteristic of solid tumors is the pathological recruitment of immunosuppressive myeloid cells, which in certain tumors includes dendritic cells (DCs). DCs are of particular interest in the field of cancer immunotherapy because they induce potent and highly specific anti-tumor immune responses, particularly in the early phase of tumorigenesis. However, as tumors progress, these cells can be transformed into regulatory cells that contribute to an immunosuppressive microenvironment favoring tumor growth. Therefore, controlling DC phenotype has the potential to elicit effective anti-tumor responses while simultaneously weakening the tumor's ability to protect itself from immune attack. This review focuses on the dual nature of DCs in the tumor microenvironment, the regulation of DC phenotype, and the prospect of modifying DCs *in situ* as a novel immunotherapeutic approach.

Keywords: myelopoiesis, dendritic cell, tumor microenvironment, immune suppression, *in situ* vaccination, cancer immunotherapy

INTRODUCTION

Accumulated experimental and clinical evidence indicate that the immune system recognizes neoplasms and attempts to mount a response against these altered cells. However, immune pressure against established tumors is clearly not sufficient to defend tumor-bearing hosts from disease progression, and eventually death. A universal occurrence in established tumor-bearing individuals is a profound alteration of myelopoiesis (1). Pathological myeloid differentiation leads to the expansion of a heterogeneous population of immunosuppressive myeloid cells that accumulates in the spleen and gives rise to regulatory macrophages and dendritic cells (DCs) in tumors (2). This diverse mix of pathological myeloid cells at different stages of differentiation (generically termed Myeloid-Derived Suppressor Cells, or MDSCs) is highly immunosuppressive (1, 3). MDSCs also contribute to enhanced angiogenesis (4), as well as the formation of metastatic niches for malignant dissemination (5, 6). Additionally, defective development alters the critical function of myeloid cells that, under normal physiological conditions, would terminally differentiate into DCs, macrophages, or neutrophils. Defective myleopoiesis results in a significant defect in antigen presentation, which is aggravated during malignant progression, and drives T cell-intrinsic transcriptional programs that promote T cell anergy and exhaustion. In contrast, certain tumors mobilize excessive amounts of lineage-committed, classical $CD11c^+$ DCs that, rather than driving tumor antigen-specific responses, impair T cell effector function at the tumor bed. Here, we will review how pathological myelopoiesis and tumor microenvironmental networks progressively abrogate the immunostimulatory function of DCs, resulting in unresponsive T cells and prevention of the lingering immune pressure exerted by remaining tumor-reactive lymphocytes. We will conclude by discussing potential approaches to overcome these effects *in vivo* and *in situ*.

TUMORS PROMOTE DEFECTIVE DC DIFFERENTIATION AND MATURATION

Dendritic cells originate in the bone marrow from the differentiation of hematopoietic precursors to Common Myeloid Progenitors and subsequently to DCs. Recent evidence indicates that, at least in mice, precursors of conventional DCs specifically express Clec9a and represent an independent lineage much less dependent on inflammation-induced monocyte differentiation than previously thought (7).

A hallmark of virtually all solid tumors is aberrant expansion of pathologically differentiated myeloid leukocytes (see **Table 1**). These cells arise from myeloid progenitors under the influence of inflammatory signals (8) and accumulate at splenic, lymphatic, and tumor locations. While they retain an immature phenotype, these MDSCs are highly immunosuppressive (9). Elegant experiments based on transfer of tumor-derived MDSCs of the myelomonocytic lineage have shown that under the influence of hypoxia in the tumor microenvironment (TME), these altered precursors of macrophages and DCs still reach their terminally differentiated cell fates (2). However, as expected from the succession of non-physiological signals that these myeloid cells receive, their phenotype is quite different from canonical macrophages and DCs generated under steady state conditions (see **Figure 1**). Tumor-differentiated macrophages, for instance, retain an immunosuppressive phenotype that contributes to accelerate malignant progression.

Table 1 | Phenotypic features of different tumor-infiltrating myeloid cell populations.

Cell type	Other names	Surface markers	Chemokine receptors	Phenotype	Human tumors observed within	Reference
MDSC		$CD11b^+GR\text{-}1^+$ (m), $Lin\text{-}CD33^+MHC\text{-}II^-$ or $CD11b^+CD33^+CD14^-$ (h)		Immunosuppressive	Breast, renal-cell, pancreatic, melanoma, head and neck	Gabrilovich and Nagaraj (9)
Mature DC	Classical DC	$CD11c^+MHC\text{-}II^{high}$	CCR7, CXCR4	Immunostimulatory		
Immature DC		$CD11c^+MHC\text{-}II^{low}$	CCR6, CCR2, CXCR4	Antigen uptake, Immunosuppression	Ovarian, breast, lung, colorectal, melanoma, renal-cell, prostate	Chaux et al. (10), Pinzon-Charry et al. (11), Perrot et al. (12)
Pre-DC		$CD11c^+MHC\text{-}II^-$	CCR1, CCR5, CCR2	Committed to DC lineage		
Regulatory DC	Tolerogenic DC	$CD11c^+CD11b^{\pm}$, $MHC\text{-}II^+CD86^{high}PD\text{-}L1^+$	CXCR4, CCR6	Immunosuppressive	Cervical, hepatocellular, breast, ovarian	Lee et al. (13), Ormandy et al. (14), Pinzon-Charry et al. (15), Scarlett et al. (16)

Mouse and human markers are indicated by (m) and (h), respectively.

In dissecting the role of DCs in cancer, most studies have focused on impaired DC differentiation as the cause of diminished production of mature, functionally competent DCs (1, 19). In support of this concept, a decrease in the accumulation of mature DCs has been found in patients with cervical (13), hepatocellular carcinoma (14), lung (12), colorectal (10), and breast cancer (15). Blockade of DC differentiation in tumor-bearing hosts was primarily attributed to VEGF, a common tumor microenvironmental factor widely known for its role in promoting tumor angiogenesis (20, 21). Accordingly, VEGF levels negatively correlate with the number of DCs in the blood or tumor in a variety of human cancers (22–24). These studies implied that the paucity of functionally mature DCs in tumors was a major contributing mechanism to overall immune evasion.

The consequence of defective antigen presentation in cancer individuals is T cell unresponsiveness, which may be the result of anergy or exhaustion. Unlike replicative senescence, anergy and exhaustion are reversible processes that result from different transcriptional programs but are frequently confused (25, 26). Anergy takes place at the time of priming, while exhaustion occurs in previously activated T cells undergoing repeated exposure to suboptimal amounts of antigen in the presence of negative costimulation. As emerging clinical evidence using PD-1 inhibitors suggests, T cell exhaustion is a major driver of tumor-induced immunosuppression in more than a third of cancer patients (27, 28).

TUMORS ALSO INDUCE THE ACCUMULATION OF FULLY COMMITTED DCs WITH IMMUNOSUPPRESSIVE ACTIVITY

While impaired DC differentiation and maturation may explain the myeloid phenotypes found in some tumors, they do not explain the accumulation of DCs with regulatory phenotypes that we and others have found in certain tumors (16, 29). Thus, we showed that classical DCs with immunosuppressive activity, termed regulatory DCs, accumulate in the TME. In tumors, these DCs suppress T cell effector functions through multiple mechanisms that include the expression of PD-L1, the production of L-Arginase and the up-regulation of tolerogenic butyrophilins (16, 30–38). The contribution of classical DCs to tumor-induced immunosuppression is therefore different from the mere lack of fully differentiated, immunostimulatory DCs, at least in certain carcinomas (see **Table 1**).

Because the myeloid leukocytes that are found in the TME represent a heterogeneous mix of abnormal cells at different stages of differentiation, phenotypic overlap and variability between patients hinder a conclusive categorization of macrophages vs. differentiated DCs vs. more immature precursors across tumor specimens. Nevertheless, we have demonstrated that the predominant population infiltrating solid human ovarian cancer specimens (but not human ascites) exhibits predominant determinants of DCs, including CD11c, HLA-DR, and DEC205, but do not express the monocyte/macrophage markers CD11b or CD14 in at least 1/3 of patients (16). Irrespective of nomenclature in human tumors, we have also demonstrated that the counterpart of this population in ovarian cancer mouse models can be induced to process full-length OVA *in vitro* (30) and *in vivo* (32, 34), and effectively present processed SIINFEKL to T cells in response to certain activating signals. These $CD11c^+$ cells also produce Zbtb46 transcripts (39, 40) and express Clec9a (7) further implying their DC nature. DCs are therefore important players of the immunosuppressive networks orchestrated by at least some frequent epithelial tumors, and defective antigen-presenting activity contributes to the abrogation of the protective function of anti-tumor T cells.

We initially assumed that these DCs were "immature," and therefore simply unable to prime T cell responses. However, ovarian cancer DCs express significant levels of CD86. Even more surprisingly, human tumor DCs in multiple specimens also express CD83, an activation marker. Furthermore, these DCs produce high levels of inflammatory cytokines such as IL-6 and the chemokine CCL3 (32–34). Additionally, although TLR activation can further up-regulate MHC-II, these DCs express relatively high MHC-II levels in the TME, in both humans and mice (16, 37). Most importantly, progressive weakening of anti-tumor

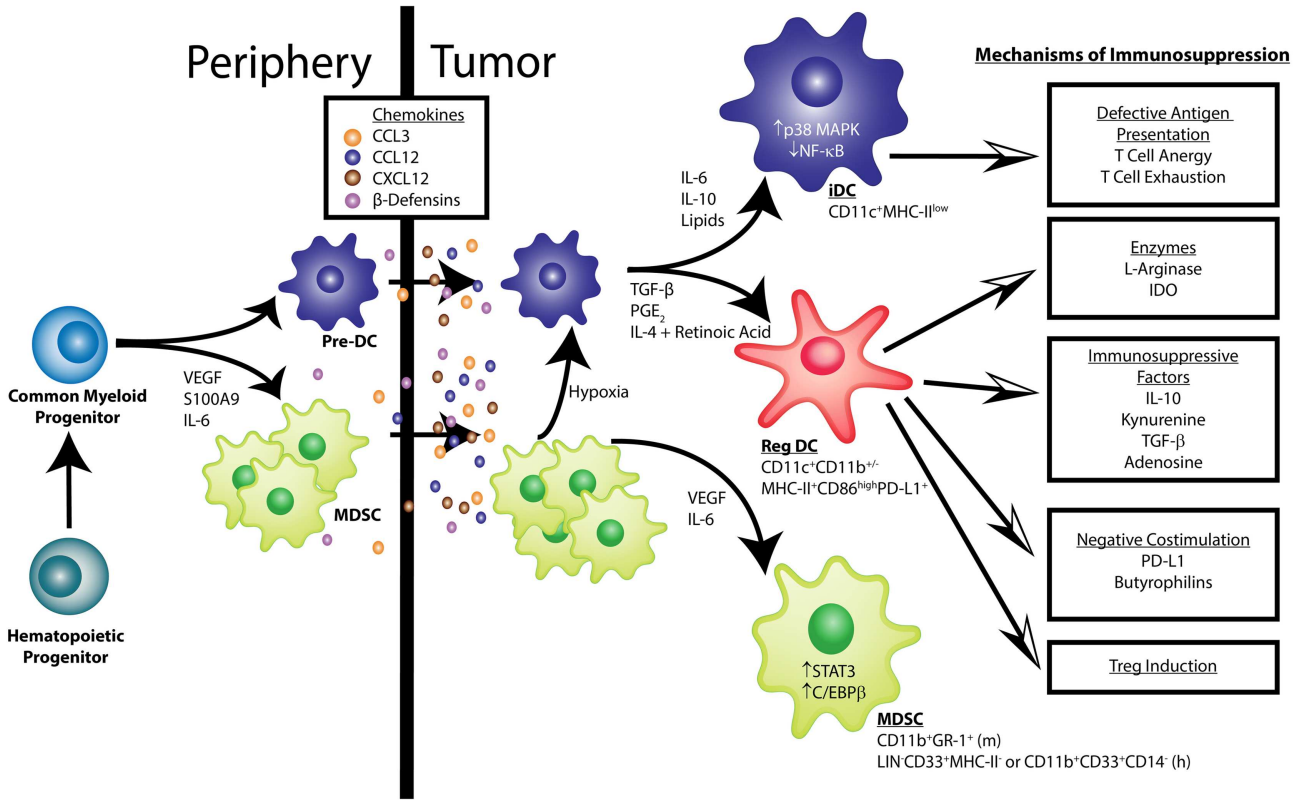

FIGURE 1 | Pathological dendritic cell differentiation contributes to tumor-induced immune evasion. Cancer-associated inflammation up-regulates the production of myeloid cells from hematopoietic progenitors in the bone marrow. Common myeloid progenitor cells give rise to pre-dendritic cells (pre-DCs) and, under the influence of tumor-derived factors (e.g., VEGF, IL-6, and S100A9), myeloid-derived suppressor cells (MDSCs). These myeloid cells migrate into the tumor microenvironment in response to chemokines such as CCL3, CCL12, CXCL12, and β-Defensins. VEGF and IL-6 activate STAT3 and C/EBPβ signaling in MDSCs, keeping them in an immature phenotype characterized as $CD11b^+Gr\text{-}1^+$ in mice and $LIN^-CD33^+MHC\text{-}II^-$ or $CD11b^+CD33^+CD14^-$ in humans. Additionally, IL-6, IL-10, and the accumulation of lipids can activate p38 MAPK signaling to prevent the acquisition of classic DC function. Also, some MDSCs can differentiate into DCs in the hypoxic environment of the tumor. Immature DCs (iDCs) exhibit low NF-κB activation, express CD11c in mice and humans, and have low MHC-II levels. These iDCs are defective antigen presenters, which induces T cell anergy and exhaustion. In other conditions, like those found in epithelial ovarian cancer, pre-DCs mature into cells that express markers of conventional DCs ($CD11c^+$ $MHC\text{-}II^+CD86^{high}$), but exert immunosuppressive functions, termed regulatory DCs (Reg DCs). Factors such as TGF-β, PGE_2, IL-4, and retinoic acid have been shown to promote this altered maturation. These Reg DCs differ from conventional DCs in their ability to suppress effector T cell function through multiple mechanisms, which include: (1) secretion of the enzymes L-Arginase and IDO that result in the depletion of essential amino acids and production of the tolerogenic metabolites adenosine and kynurenine; (2) release of immunosuppressive factors such as IL-10 and TGF-β; (3) expression of costimulatory surface molecules, including PD-L1 and butyrophilins, that negatively regulate anti-tumor T cells; (4) induction of regulatory T cells (Tregs). More information regarding the source of the various secreted factors that govern the accumulation and function of tumor-associated myeloid cells can be found in recent reviews by Hanahan and Weinberg as well as Lindau et al. (17, 18).

immunity cannot be solely attributed to "scarcity" of mature DCs in these tumors because depleting DCs at advanced stages of malignant progression in preclinical models paradoxically delays tumor growth, rather than being simply "neutral" (16). Excessive accumulation of immunosuppressive DCs, rather than mere absence of immunostimulatory antigen-presenting cells (APCs), is therefore the predominant mechanism of DC dysregulation in at least ovarian carcinoma.

These regulatory DCs are also different than their immature precursors due to their main location of action. Immature DCs that fail to efficiently activate T cells in the lymph node will primarily prevent T cell priming, leading to anergy or tolerization. And while we have identified immunosuppressive, regulatory DCs in the draining lymph node (16), the remarkable suppression by tumor-infiltrating DCs contributes to a protective barrier for tumor cell growth. By suppressing effector T cells through many mechanisms we discuss here, tumor-infiltrating DCs can effectively shut down activated anti-tumor immune responses. This important difference has imperative consequences for the fate of therapies that rely solely on eliciting tumor-directed T cells. For this reason, we mostly focus on the action of these altered DCs inside the tumor in this review.

Based on our converging evidence, we propose that regulatory DCs in tumors are not immature, but acquire an alternative phenotype in response to a different transcriptional program. Some peculiarities of this aberrant program have been identified in our recent studies. Thus, we demonstrated that delivery of synthetic (and functional) microRNA-155 (miR-155) specifically

to $CD11c^+DEC205^+MHC\text{-}II^+$ DCs in the microenvironment of ovarian cancer-bearing mice induced genome-wide transcriptional changes that were sufficient to transform these immunosuppressive leukocytes into an immunostimulatory cell type (37). Compared to the delivery of non-targeting double-stranded RNA of similar length and structure, ectopic miR-155 induced silencing of multiple immunosuppressive mediators, including *Tgfb1* and *Cd200*. As expected, miR-155 also targeted crucial transcription factors involved in aberrant myeloid differentiation in tumor-bearing hosts, including *Cepb/*β and *Socs1*. Most importantly, miR-155 activity down-regulated *Stat3* and, interestingly, *Satb1*, a master genomic organizer (37, 41). Unexpectedly, however, expression of miR-155 also silenced the expression of *Pu.1/Sfpi1* and *Irf8* (>150-fold in both cases) (37). This is significant because PU.1 promotes DC differentiation by inducing the remodeling of the higher-order chromatin structure at the *Irf8* gene. Therefore, myeloid IRF8 expression depends on high PU.1 levels (42). Most importantly, DC commitment requires active IRF8 to avoid alternative myeloid-lineage differentiation (42). The fact that synthetic miR-155-expressing DCs acquire the capacity to effectively present antigens *in vivo* and *in vitro* suggests that they were fully committed to a DC phenotype, albeit transcriptionally different from their classical immunostimulatory counterparts.

TUMOR- AND STROMA-DERIVED CHEMOATTRACTANTS DRIVE DC RECRUITMENT TO TUMOR LOCATIONS

The presence of regulatory DCs, as opposed to simply immature precursors, in the microenvironment of different tumors can be partially driven by the abundance and repertoire of tumor- and stroma-derived chemokines and cytokines, which is dependent on differential DC expression of chemokine receptors (43–45). For instance, the chemokine CCL3, aberrantly up-regulated in many tumors, enhances pre-DC recruitment in both *in vitro* and *in vivo* models of melanoma, colon, and lung carcinoma. Moreover, CCL3 preferentially recruits pre-DCs to tumor locations, as antibody mediated neutralization of CCL3 does not result in a decrease in any other $CD45^+$ leukocyte subset, nor does it alter the frequency of splenic pre-DCs (46). Furthermore, pre-DC expression of CCR1 and CCR5 provide a mechanism for CCL3-mediated recruitment (47). In addition, tumor hypoxia, which characterizes a wide variety of TMEs, also triggers DC and immature myeloid cell recruitment via tumor-derived CCL12.

Additionally, tumor microenvironmental prostaglandin E_2 (PGE_2) up-regulates the chemokine CXCL12, thereby accumulating CXCR4-expressing regulatory DCs (48, 49). Another relatively uninvestigated factor that promotes the recruitment of $CCR6^+$ regulatory DCs to tumor locations is the expression of β-Defensins by epithelial cells and inflammatory leukocytes. In preclinical models, ectopic tumor expression of *Defb29* has been shown to accelerate the accumulation of DCs in the TME, leading to more aggressive malignant progression (30). Defensins are clearly powerful chemoattractants (50) that are expressed at significantly higher levels in certain cancer patients (51).

Perhaps the most compelling evidence that tumor-derived chemokines influence the type of DCs found in the tumor has been demonstrated in a renal-cell carcinoma model (52), where the intra-tumoral or peri-tumoral distribution of DCs is determined by CCL20 and CCL19 levels. High intra-tumoral levels of CCL20 result in the accumulation of immature $CCR6^+$ DCs within the tumor bed, whereas high peri-tumoral levels of CCL19 promote the accumulation of mature $CCR7^+$ DCs preferentially to the tumor margin.

PATHWAYS PREVENTING DENDRITIC CELL DIFFERENTIATION

The accumulation of immature DCs and earlier progenitors are also the result of corrupted pathways of inflammation-driven myeloid differentiation. The extent of the corrupted nature of myelopoiesis in cancer has been underscored by recent studies by Gabrilovich and colleagues showing that the epigenetic silencing of the retinoblastoma gene in immature (non-tumor) myeloid cells is sufficient to drive transdifferentiation of myelomonocytic MDSCs to granulocytic MDSCs (53). A block in the differentiation of immature myeloid precursors into mature myeloid cells results in fewer DCs and an accumulation of MDSCs. The molecular pathways driving this process are known to be STAT3-dependent. For instance, high STAT3 activity has been shown to inhibit both the differentiation of mature DCs from myeloid precursors (54) and the activation and MHC-II up-regulation of DCs (55). Still, the molecular mechanism of this STAT3 signaling remains incompletely understood. Studies by Lee and colleagues have shown that tumors mediate both STAT3 activation and PKC βII down-regulation in DC progenitor cells (56). Importantly, PKC βII repression can be mimicked by the expression of a constitutively active STAT3 mutant. Because PKC βII is required for DC differentiation (57), these reports have identified a roadblock for subsequent maturation of myeloid precursors, but more studies are needed to fully dissect this mechanism.

Many factors overexpressed in the tumor macro- and microenvironments promote STAT3 activation. It has been demonstrated that the negative effect of tumor conditioned media on DC activation could be reversed by using either STAT3 null DCs or DCs treated with a peptide inhibitor of STAT3 (58). Some mediators of this process include IL-6, IL-10, and VEGF, which are generally increased systemically in cancer patients and negatively correlate with prognosis (59). The STAT3 activator IL-6 is particularly important for the development of functionally defective DCs (60). Tumor-derived IL-6 drives STAT3 nuclear translocation in immature myeloid cells, thus promoting proliferation and inhibiting apoptosis of MDSCs. Furthermore, IL-6 and M-CSF secreted by human renal carcinoma cell lines inhibit DC differentiation from $CD34^+$ bone marrow progenitors (61). In addition, STAT3 up-regulates S100A9 in hematopoietic progenitor cells, which inhibits further differentiation to DCs and retains these cells as MDSCs, leading to tumor tolerance (62).

Besides STAT3, some of these cytokines inducing MDSCs also act on a common molecular pathway that is entirely dependent on the C/EBPβ transcription factor. C/EBPβ is therefore required for the immunoregulatory activity of both tumor-induced and bone marrow-derived MDSCs (63).

Additionally, VEGF has been shown to impair DC differentiation from progenitors through the inhibition of another pathway that is also important for DC maturation; namely, by preventing NF-κB activation (21, 64).

PATHWAYS PREVENTING DENDRITIC CELL MATURATION

Besides impairment of DC development, DCs can be found in an active immature phenotype associated with induction of peripheral tolerance. Numerous studies have demonstrated that these immature DCs (iDCs) are unable to support normal levels of antigen-specific T cell expansion. For example, human monocyte-derived DCs (mo-DCs) are unable to induce expansion of antigen-specific, tetramer-positive T cells when kept in an immature phenotype by differentiation in the presence of the adenosine receptor agonist NECA or cAMP elevating agents FSK/IBMX (65).

Under steady state conditions, DCs exist in an immature state characterized by high phagocytic activity and low antigen-presenting capabilities. Upon being activated by innate pathogen or damage associated signals, DCs acquire a mature state characterized by MHC presentation of antigens and costimulatory signaling via B7 family molecules (e.g., CD80 and CD86). For an immature DC to become an efficient APC, it must receive specific activating signals through receptors such as CD40, TNF-R, IL-1R, and TLRs (66). Although the intricacies of how DC maturation is blocked remain to be fully dissected, an important mechanism preventing DC maturation appears to be mediated by the p38 MAPK pathway. IL-6, IL-10, and TGF-β contained in myeloma-conditioned media have been shown to activate this pathway and prevent the immunostimulatory activity of immature DCs. Abrogating p38 MAPK with a small molecule inhibitor during DC development enhanced their subsequent activation, making them more mature and stimulatory even when differentiated in the conditioned media (67). In addition, paclitaxel-induced inhibition of p38 MAPK activity decreases the production of S100A9 and TNF-α by MDSCs, resulting in reduced tumor burden and increased animal survival (68). In tumor-bearing mice, paclitaxel induces the maturation-dependent antigen-presenting activity of DCs (69).

High levels of IL-10 in tumor-bearing hosts also impair the complete maturation of DCs. DCs from mice that overexpress IL-10 have low expression of MHC-I and costimulatory molecules and are deficient at stimulating T cell responses (70). Accordingly, IL-10 treated human DCs induce anergy in T cells, although fully matured DCs were resistant to the effect of IL-10 (71). Pancreatic tumor cells were also found to secrete IL-6 and IL-10 thus suppressing the stimulatory abilities of DCs in allogeneic reactions (72).

Another pathway relevant for effective DC maturation is dependent on NF-κB activation. Signaling via NF-κB is required for the professional APC function of DCs, including high expression of MHC-II and costimulatory molecules, secretion of IL-12 and TNFα, and stimulation of allogeneic T cells (73). Recently, PD-1 expression on DCs from murine ovarian tumors was found to decrease NF-κB activation, resulting in less production of inflammatory cytokines (74).

Additional mechanisms that remain poorly understood but could be very important for preventing the immunostimulatory potential of tumor-associated DCs are dependent on how myeloid leukocytes metabolize lipids in the TME. Tumor DCs up-regulate scavenger receptor A (SR-A), a target used for therapeutic depletion of regulatory DCs in preclinical models (75). Overexpression of SR-A increases the uptake of extracellular lipids, which are pathologically accumulated in DCs (76). Tumor DCs from mice and humans were demonstrated to have relatively high levels of triglycerides that impaired their ability to process antigen, and could be functionally restored upon normalization of their triglyceride levels.

Finally, immunomodulatory signals can also come from metabolites, as tumor-derived lactate can skew the differentiation of human monocytes into less mature DCs (defined by less CD1a expression) that are deficient in their ability to stimulate T cells and secrete IL-12 (77).

PATHWAYS DRIVING THE TRANSFORMATION OF DENDRITIC CELLS INTO AN IMMUNOSUPPRESSIVE CELL TYPE

In addition to blocking DC differentiation and full activation, the TME also contains multiple factors that transform classical DCs with antigen-presenting capabilities into immunosuppressive players. Our recent studies in a preclinical model of sarcomatoid carcinoma in immunocompetent mice have illustrated that this switch takes place during malignant progression (16, 37). In this system, the inflammatory microenvironment of advanced tumors recapitulates the molecular and cellular components of immune cells found in human solid tumors. We found that at initial stages of tumor development, DCs elicit T cell responses that are able to put tumor growth in check for relatively long periods. As expected, depleting DCs at early stages of tumor progression resulted in accelerated tumor progression, which mimicked T cell depletion. However, as these tumors advanced, DC differentiation was not blocked. Rather, $CD11c^{+}MHC\text{-}II^{+}DEC205^{+}$ DCs with regulatory activity started accumulating at tumor locations, which coincided with the beginning of the exponential growth phase of these latent tumors. Strikingly, DC depletion at this advanced stage of malignant growth was sufficient to significantly delay (rather than advance) malignant progression. DCs in these late-stage tumors are therefore not simply immature or unable to effectively present antigens. In fact, although DCs in advanced tumors exhibited lower expression of MHC-II and costimulatory CD40, they still expressed both at significant levels. Instead they became active accomplices to tumor growth through the inhibition of protective immunity.

Dendritic cell accumulation in solid tumors is not only found in the microenvironment of solid ovarian carcinomas. For instance, Norian et al. found that $MHC\text{-}II^{+}CD11b^{+}CD11c^{high}$ DCs infiltrating established mammary carcinomas could also act as regulatory players by inhibiting CD8 T cell function through L-Arginase production, thus dampening T cell-mediated anti-tumor immune protection (29). Naïve T cells primed with these DCs undergo minimal expansion and defective IFN-γ production, and eventually become anergic. The phenotype of these regulatory DCs therefore does not correspond to the plasmacytoid DC type that has been traditionally associated with the development and maintenance of immunosuppression, although these cells are also found in the microenvironment of many tumors (78–81).

Among the potential tumor microenvironmental factors driving the transformation of immunostimulatory DCs into immunosuppressive players, we identified that at least PGE_2 and TGF-β (but not IL-6) in supernatants from primary cultures, are both necessary to elicit a regulatory phenotypic switch in DCs

from early tumors. These DCs were then capable of suppressing the strong proliferation of tumor-reactive T cells in response to tumor antigen presented by other DCs (16, 37).

In addition, IL-4 and retinoic acid synergize to induce the expression of *Aldh1a2* in GM-CSF-differentiated inflammatory DCs, turning on their regulatory activity (82). Retinoic acid is also known to enhance TGF-β-induced Smad3 activation (83), potentially synergizing with the induction of suppressive features elicited by TGF-β on DCs (16, 37).

Another important factor dampening the immunostimulatory potential of tumor DCs is kynurenine, the first product of the tryptophan degradation pathway generated by Indoleamine-pyrrole 2,3-dioxygenase (IDO) (84, 85). Kynurenine, by interacting with the aryl hydrocarbon receptor (AHR), elicits an autocrine loop in DCs, resulting in enhanced IDO activity and the acquisition of an immunosuppressive phenotype by originally immunocompetent DCs (86). In another study, IDO expression was found to be required for DC-induced tolerance, and, via TGF-β-induced expression of IDO, it was possible to convert CD8-negative DCs from being immunogenic to regulatory (87).

MECHANISMS OF IMMUNOSUPPRESSION DRIVEN BY DC SECRETION OF IMMUNOSUPPRESSIVE FACTORS

Regulatory DCs suppress immune responses by secreting anti-inflammatory soluble factors that inhibit effector T cell functions or skew T cell responses. DCs, for instance, are a major source of IDO within the TME. DCs infiltrating multiple tumors show enhanced IDO activity. For example, IDO-expressing FOXO3$^+$ DCs were shown to promote malignant progression in preclinical models of prostate cancer (88). Kynurenine produced by IDO activates AHR, which is central to T cell differentiation into FoxP3$^+$ regulatory T cells (Tregs) (89, 90). Generation of induced Tregs is not only a property of murine tumor DCs, as IDO expression in human DCs also results in induction of Foxp3$^+$, immunosuppressive T cells when these DCs are co-cultured with healthy donor CD3$^+$ lymphocytes. This induction was confirmed to be IDO-dependent by reversal of T cell phenotype following tryptophan treatment (91).

Regulatory DCs, and not only macrophages or MDSCs, are also important contributors to immunosuppression in the TME through the production of L-Arginase (16, 29, 37). L-Arginase activity results in catabolic depletion of Arginine, an amino acid essential for effector T cells. Upon undergoing a phenotypic switch from being immunostimulatory to immunosuppressive, DCs found in advanced tumors significantly increase L-Arginase activity. Importantly, freshly dissociated human ovarian carcinoma specimens also contain DCs with significant L-Arginase activity (16, 37).

Among the factors that enhance L-Arginase activity in the TME, IL-6 is perhaps the best characterized. IL-6 treatment of bone marrow-derived DCs (BMDCs) results in increased L-Arginase RNA, protein, and activity both *in vitro* and *in vivo*. The consequent drop in extracellular arginine resulted in down-regulation of MHC-II in the same DCs and impaired ability to activate OT-II T cells. These results were similarly reproduced by differentiating BMDCs in arginine-free conditions (92).

Other immunosuppressive factors produced by DCs in advanced tumors include cytokines such as TGF-β and IL-10. Tumors induce DCs to secrete TGF-β, further promoting Treg expansion and indirectly suppressing T cell effector functions (93, 94). However, the relative contribution of DC-derived TGF-β compared to the production of this cytokine by other microenvironmental cell types (e.g., certain T cell subsets, including Tregs), needs to be comprehensively addressed.

MECHANISMS OF IMMUNOSUPPRESSION DRIVEN BY DCs THROUGH MEMBRANE-BOUND DETERMINANTS

In addition to not providing sufficient antigen or costimulation, tumor-associated DCs frequently express negative costimulatory molecules that suppress T cell activity. As evidenced by the emerging success of novel clinical inhibitors, perhaps the most important negative signaling is mediated through PD-L1:PD-1 interactions (95). PD-1 is a negative costimulatory receptor primarily expressed on activated lymphocytes. The most abundant ligand for PD-1, B7-H1/PD-L1, is up-regulated in DCs and tumor cells in multiple cancers. PD-1 itself can also be expressed by tumor DCs themselves, at least in murine models. Expression of the PD-L1:PD-1 pair was found to increase throughout malignant progression, correlating with loss of positive costimulatory markers (CD80, CD86, and CD40), a lack of cytokine release (IL-12, IL-10, IL-6, TNFα, and G-CSF), and contact-dependent inhibition of T cell expansion (74). Importantly, inhibitors for both the ligand (PD-L1) and the receptor (PD-1) have been developed and have shown impressive clinical results (27, 28). However, PD-1 blockers appear to be better candidates for future FDA approval, while PD-L1 inhibitors appear to produce better results in murine models (96), possibly related to the affinity and pharmacokinetics of different humanized and mouse-specific antibodies.

Aside from PD-L1, regulatory DCs can express other negative costimulatory molecules. One of these inhibitors, universally expressed in ovarian cancer-infiltrating DCs, is CD277 (35). CD277 identifies various highly similar members of the butyrophilin subfamily 3 (BTN3). The function of these molecules is poorly investigated, but they share sequence and structural homology to the negative costimulatory molecule B7-H4. CD277 is expressed by CD45$^+$ MHC-II$^+$ APCs isolated from human epithelial ovarian cancer samples, and is up-regulated in human mo-DCs in response to molecules found in the TME, such as IL-6, IL-10, VEGF, PIGF-1, and CCL3. We showed that CD277 expressed in artificial APCs consistently decreased the expansion of TCR-stimulated T cells. However, one of the butyrophilins expressing the CD277 epitope (BTN3A1), has been recently reported as an activating receptor (thus not a ligand) in γδ T cells, where it binds phosphorylated antigens with low affinity (97). In addition, other similar molecules such as BTNL8 have been associated with immunostimulatory activity when a soluble fusion protein was used (98). It is unclear, however, whether the activity of butyrophilins depends on engagement of the unknown receptor in T cells in a cross-linked or soluble form.

Also important for the generation of tolerogenic mediators are the two ecto-enzymes CD39 and CD73 that act sequentially to generate anti-inflammatory extracellular adenosine (72, 99, 100). Among the many suppression-promoting effects that

TGF-β induces in DCs, it up-regulates the expression of CD73 (101). CD73 produces adenosine from AMP, which engages with the adenosine A2A receptor (A2AR). A2AR ligation both inhibits the expansion of effector T cells and promotes the generation of induced Tregs (102). Chemical enzymatic inhibitors or neutralizing antibodies targeting these ecto-enzymes therefore offer novel promising avenues of therapeutic interventions.

IMPLICATIONS OF DC DYSREGULATION IN CANCER PATIENTS FOR THERAPEUTIC DENDRITIC CELL-BASED VACCINES

Because DCs are the most potent APCs, they have been used to boost T cell-mediated immune protection against cancer for nearly two decades. Multiple approaches have been shown to work effectively in mice, primarily as prophylactic interventions. However, any reproducible clinical benefit for patients with established cancer has been marginal so far (103). It is true that there is still room for improvement, especially regarding immunostimulatory cell type and route of administration. However, converging clinical evidence suggests that quantifiable improvements in immunological readouts are not associated with reproducible clinical responses (104).

Although DC-based vaccines are designed to overcome defective maturation, challenges with migration to places of T cell priming and, especially, the abrasive effect of the immunosuppressive networks in the TME, have so far rendered vaccine-induced T cell responses ineffective against tumor-induced tolerance. A successful trial using autologous lysate-pulsed DC vaccination in recurrent ovarian cancer has become an auspicious exception (105). However, DC vaccination in this trial was followed by adoptive transfer of vaccine-primed, *ex vivo* stimulated T cells, and it is therefore unclear whether the obvious clinical responses can be at all attributed to the initial vaccine. Nevertheless, this approach opens new avenues for the use of DCs for effective priming of autologous tumor-reactive T cells *ex vivo*.

All the aforementioned mechanisms of DC dysfunction in advanced malignancy contribute to our understanding of the failure of DC vaccines to deliver on their original promise. Even if *ex vivo* matured DCs could reach lymph nodes and can effectively prime tumor antigen-specific T cells without being affected by TFG-β, retinoic acid, or IDO metabolites, it is becoming increasingly clear that anti-tumor T cells will succumb in the TME, unless immunosuppression is concurrently targeted. Consequently, new opportunities emerge from the use of DC-based vaccines to treat early-stage disease, where they can be more efficacious in the absence of systemic immune dysfunction (106). DC-based vaccines, for instance, are now being tested against ductal carcinoma *in situ* (DCIS), to prevent development of subsequent breast cancer. Recent trials observed reduced recurrences in patients with estrogen receptor negative DCIS. Because the immunosuppressive networks are not as strong at this disease stage, this approach may be more promising (107).

REVERSING THE PHENOTYPE OF DENDRITIC CELLS FROM AN IMMUNOSUPPRESSIVE TO AN IMMUNOSTIMULATORY CELL TYPE

While the establishment of regulatory DCs is a significant pathological event in solid tumors, the central role that DCs play in orchestrating adaptive immunity still offers opportunities for therapeutic intervention that could have lasting benefit. *In situ* vaccine-based interventions aimed at transforming tumor DCs into activated APCs capable of priming host anti-tumor T cells represent a promising approach to both actively boost T cells and inhibit immunosuppression. Vicari and colleagues first reported that tumor-infiltrating DCs are able to be rescued and become effective tumor antigen presenters in the context of MHC-I, provided that they receive the right stimulatory signals. They found that DCs were refractory to stimulation with the combination of LPS, IFN-γ, and anti-CD40 antibody, but tumor-induced DC paralysis could be reverted by a combination of CpGs and an anti-IL-10R antibody (108).

The combination of signals promoting the immunostimulatory capacity of otherwise immunosuppressive DCs may depend on different tumor settings. The use of agonistic anti-CD40 antibodies as a single intervention has been successful at activating tumor DCs to stimulate T cell rejection of a murine tumor model (109). In addition, a fully human CD40 agonist antibody, CP-870,893, has been tested in humans with advanced cancers, resulting in objective responses in 14% of patients (110). However, mechanistic studies in preclinical models identified macrophages as direct mediators of cytolytic anti-tumor activity, with negligible contribution of anti-tumor T cells (111). Another trial using weekly dosing of CP-870,893 in advanced cancer patients showed only stable disease as the best clinical response. Some patients showed decreased T cell numbers, indicating that the dosing interval may have been too frequent (112). In our preclinical systems or in human tumor-derived DCs, CD40 agonists alone had no measurable effect on DC activation *in vivo* (34). However, based on the optimization of multiple combinations of vaccine adjuvants carried out by Ahonen et al. (113), we confirmed that CD40 and TLR3 agonists synergize to transform ovarian cancer-associated DCs from an immunosuppressive to an immunostimulatory cell type, both *in vivo* and *in situ* (34). These findings indicate the importance of acknowledging differences in the TME among various cancers, including that treatments like CD40 agonists may act on cells other than DCs. While in some tumors, CD40 agonists may singlehandedly activate DCs, in other tumors, like ovarian carcinoma, combining CD40 agonists with TLR activation will be necessary to revert DCs from immune-suppressors into functional APCs.

The other component of our synergistic combination, TLR agonists, can also activate DCs and has been tested in cancer with some success as a monotherapy. TLR9 agonists, for instance, have been developed and are in clinical trials (114). Stronger stimulation of DCs can be achieved through the activation of TLR3 with poly(I:C). To address its undesirable toxicity in humans, a less stable version of poly(I:C) called poly(I:C_{12}U) was developed by incorporating a mismatched uracil, which still functionally activates DCs and enhances their IL-12 production (115).

Recent studies from our group have also underscored the potential of immunostimulatory nanoparticles carrying functional RNA against tumors that are compartmentalized, such as ovarian cancer. We showed that nanocomplexes comprised of polyethylenimine (PEI), a biocompatible polymer and TLR5

agonist, and siRNA oligonucleotides targeting PD-L1 are selectively engulfed by DCs in the ovarian cancer microenvironment (32). Activation of multiple TLRs and PD-L1 silencing synergize to promote the capacity of DCs at tumor locations to present the tumor antigens that they spontaneously phagocytose in the TME. Taking advantage of this optimized system, we were also able to deliver immunostimulatory miR-155 specifically to immunosuppressive DCs in mouse ovarian tumors, through the use of synthetic, functional double-stranded RNA Dicer substrates (37). Augmenting miR-155 activity resulted in genome-wide transcriptional changes that transformed DCs from a regulatory to an immunostimulatory phenotype. Although human tumor ascites primarily accumulates canonical macrophages, it is plausible that miR-155 supplementation also exposes their capacity to effectively present antigens. In addition, DCs are recruited to solid ovarian cancer masses, where they accumulate at the growing edge, in contact with ascites. Intra-peritoneal delivery of immunostimulatory nanocomplexes, therefore, offers significant promise to reverse the immunosuppressive activity of ovarian cancer-associated phagocytes. These approaches, however, need to be clinically tested.

Finally, one method that harnesses the power of DCs is actually a beneficial side effect of a classic treatment: chemotherapy. Certain chemotherapies are now understood to cause an immunogenic death of tumor cells that primes DCs to activate an anti-tumor immune response. This mechanism was first described for doxorubicin, showing that it induced immunogenic, caspase-driven tumor cell death that stimulated a protective immune response dependent on DCs and CD8 T cells (116). The features of immunogenic cell death include the release of ATP (117), surface exposure of calreticulin (118), and secretion of HMGB (119), which respectively act to recruit (120), induce engulfment by (121), and activate DCs for T cell stimulation. The list of agents inducing immunogenic cell death has been extended to include anthracyclines, oxaliplatin (but not cisplatin), and irradiation, among others. For a recent comprehensive review, the reader is referred to Kroemer et al. (122).

FINAL REMARKS

Pathological myelopoiesis in cancer individuals results in the accumulation of a heterogeneous mix of MDSCs, macrophages, immature DCs, and regulatory DCs. This results in defective antigen presentation, which causes T cell anergy and, especially, exhaustion. In addition, certain tumors mobilize classical DCs with immunosuppressive activity known as regulatory DCs. All these mechanisms have hindered the success of DC-based vaccines. However, novel approaches aiming to prevent tumor recurrences at early stages or using DCs for *ex vivo* priming of tumor-reactive lymphocytes offer significant promise. Finally, the antigen-presenting capacity of tumor-infiltrating DCs can be promoted *in vivo* and *in situ*, thus achieving the double goal of reversing immunosuppression and directly boosting protective immunity.

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Clinical implications of co-inhibitory molecule expression in the tumor microenvironment for DC vaccination: a game of stop and go

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The aim of therapeutic dendritic cell (DC) vaccines in cancer immunotherapy is to activate cytotoxic T cells to recognize and attack the tumor. T cell activation requires the interaction of the T cell receptor with a cognate major-histocompatibility complex-peptide complex. Although initiated by antigen engagement, it is the complex balance between co-stimulatory and co-inhibitory signals on DCs that results in T cell activation or tolerance. Even when already activated, tumor-specific T cells can be neutralized by the expression of co-inhibitory molecules on tumor cells. These and other immunosuppressive cues in the tumor microenvironment are major factors currently hampering the application of DC vaccination. In this review, we discuss recent data regarding the essential and complex role of co-inhibitory molecules in regulating the immune response within the tumor microenvironment. In particular, possible therapeutic intervention strategies aimed at reversing or neutralizing suppressive networks within the tumor microenvironment will be emphasized. Importantly, blocking co-inhibitory molecule signaling, often referred to as immune checkpoint blockade, does not necessarily lead to an effective activation of tumor-specific T cells. Therefore, combination of checkpoint blockade with other immune potentiating therapeutic strategies, such as DC vaccination, might serve as a synergistic combination, capable of reversing effector T cells immunosuppression while at the same time increasing the efficacy of T cell-mediated immunotherapies. This will ultimately result in long-term anti-tumor immunity.

Keywords: DC vaccination, tumor microenvironment, checkpoint blockade, tumor-specific T cells, cancer treatment

INTRODUCTION

The goal of cancer immunotherapy is to activate, or reactivate, the immune system in cancer patients for therapeutic benefit. This is a challenging endeavor, as escape from immunosurveillance is an essential requirement for tumor progression. Early tumors can be eliminated or contained by the immune system but, by a process involving immunoediting, tumor cells can eventually escape this detection (1). They do so by hiding from immune detection, blocking the function of immune cells, and/or by influencing immune cells to induce tolerance to the tumor and even to produce tumor growth enhancing factors. Despite this escape from immunosurveillance, there is ample evidence indicating that it is possible to induce specific anti-tumor immune responses either naturally (spontaneous) or therapeutically. This requires a number of discrete steps. Firstly, dendritic cells (DCs) must take up and present antigens derived from the tumor, which can be encountered *in situ* or delivered to the DCs *ex vivo* as part of a therapeutic vaccine. This has to be coupled to an activation or maturation signal to the DC. Next, these mature tumor antigen presenting DCs migrate toward the lymphoid organs, where they have to induce antigen-specific T cell responses that target the tumor (2, 3). Efficient anti-tumor responses are believed to require $CD8^+$ cytotoxic (killer) T cells, but recent data indicate that induction of $CD4^+$ T helper cells also contribute to clinical efficacy (4). Conversely, DCs may also trigger antibody and natural killer (NK) cell responses, which can contribute to anti-tumor immunity (5, 6).

Priming of naïve T cells into antigen-specific effector T cells by DCs requires four signals (**Figure 1**): (I) engagement of a T cell receptor (TCR) with a peptide-major-histocompatibility complex (MHC) on the DC and (II) the right balance between expression of co-stimulatory molecules that activate T cell proliferation and co-inhibitory molecules that attenuate T cell activation on both cell types. (III) A third signal is provided by cytokines secreted by the DCs, which promote T cell differentiation and polarization toward specific effector T cell phenotypes. Finally (IV), DCs regulate the induction of specific chemokine receptors and integrins on T cells to direct migration toward specific tissues (2, 7–10).

The above-described induction of T cell-mediated anti-tumor immunity can be exploited therapeutically in several ways, the two most popular being DC vaccination strategies and adoptive T cell transfer. These intervention strategies are referred to as cell-based immunotherapy and both rely on the isolation of autologous immune cells from a patient followed by *ex vivo* manipulation and then re-infusion into the patient. In recent years, much progress

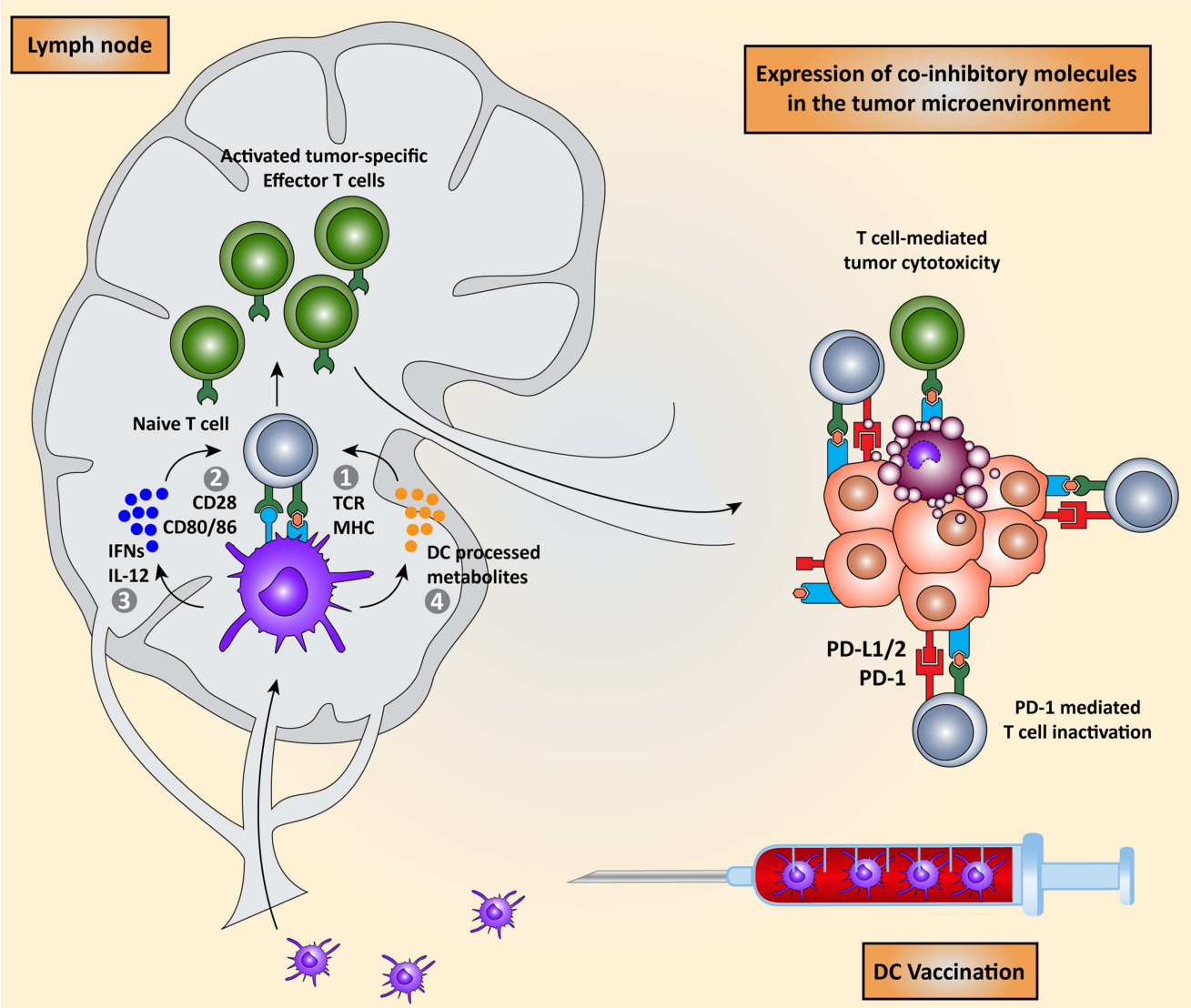

FIGURE 1 | Dendritic cell vaccination is counteracted by host immunosuppressive mechanisms. Monocytes or natural occurring dendritic cells are isolated from the peripheral blood of the patient, loaded with tumor antigens, and subsequently matured. These activated DCs are re-infused into the patient and migrate to the lymph node to encounter and interact with naïve T cells in order to induce the activation of effector T cells. DC-mediated T cell activation is regulated by four signals: (I) interaction between TCR on T cells and MHC:peptide complex, (II) co-stimulation via CD28 and CD80/86 expressed on T cells and DCs respectively, (III) secretion of pro-inflammatory cytokines such as IFNs and IL-12, and (IV) release of DC-processed metabolites. These activated $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells migrate to the tumor site where they are eventually neutralized by the immunosuppressive nature of the tumor microenvironment due, for instance, to the expression of co-inhibitory molecules.

has been made in this field: tumor antigens, DCs, and T cells, as well as adjuvants have been optimized, leading to an increase in the number of patients with an anti-vaccine immune response. However, despite these improvements, the clinical responses are still limited. This is most likely caused by the establishment of an immunosuppressive tumor microenvironment. As such, to further improve immunotherapeutic approaches, strategies to neutralize immunosuppression are required. A promising strategy, and the main subject of this review, involves the manipulation of co-stimulatory and co-inhibitory molecules to change the balance within the tumor microenvironment from an immunosuppressive state into an immunostimulatory state.

We will first discuss the current state of DC vaccination, followed by how these therapies could be affected by the immunosuppressive tumor microenvironment. Subsequently, we will review current strategies for reversing the immunosuppressive state of the tumor microenvironment, which are in clinical or pre-clinical stage. We will conclude by discussing the merits of combining DC vaccination with blockade of immune checkpoints in cancer treatment.

DENDRITIC CELL VACCINES

Dendritic cells are the most potent antigen presenting cells (APCs) and provide a key functional link between innate and adoptive immune responses. In their immature state, they take up and process antigens in the peripheral blood and tissue, then undergo maturation and migrate to lymphoid organs where they present the antigens to naïve T cells (11). These mature DCs, now expressing high levels of cell surface MHC class I and II molecules, can activate both naïve $CD8^+$ cytotoxic T cells and naïve $CD4^+$ T helper cells (12–14) in a process dependent on the upregulation of co-stimulatory molecules such as CD40, CD80, CD86, and OX40L on the APC surface (7, 15). These molecules interact with corresponding ligands expressed on T cells (**Figure 1**), with the interaction between CD86 on DCs and CD28 on T cells being the most significant to trigger T cell activation and expansion (16, 17). Conversely, T cells and DCs also express co-inhibitory molecules, such as the receptors programed cell death-1 (PD-1) and the cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) expressed on T cells and the ligands PD-ligand 1 (PD-L1) and PD-ligand 2 (PD-L2) present on DCs. The interaction between these co-inhibitory molecules can inhibit T cell priming and activation and the delicate balance between co-stimulation and co-inhibition determines the fate of a T cell response. The expression and regulation of these proteins on DCs and T cells have been recently reviewed (2). During the process of co-stimulation, DCs secrete cytokines that regulate the differentiation of naïve T cells into different subsets of effector T cells, in particular $CD4^+$ T helper cells. This process results in the differentiation toward a Th1, Th2, Th9, Th17, or regulatory T cell (Treg) phenotype (18). Lastly, environmental cues from the DCs, such as DC-processed metabolites, provide T cells with a signal to home, and migrate to certain tissues (19).

Therapeutic DC vaccination strategies against cancer aim to exploit the ability of DCs to prime antigen-specific T cells, in order to induce a T cell-mediated, specific, immune response that targets and destroys the tumor. DCs, for example naturally occurring blood DCs or *ex vivo* generated monocyte-derived DCs, are provided with tumor-specific antigens, either by loading them *ex vivo* with the tumor peptides and then injecting the cells back into the patient or by targeting them *in vivo* (3, 20–22). At first, DC vaccination protocols mainly focused on targeting cytotoxic $CD8^+$ T cells, but it has become clear that $CD4^+$ T cells not only augment the induction and proliferation of these $CD8^+$ T cells, but also participate in the elimination of tumor cells and maintenance of long-term immunity. Thus an efficient vaccine should be able to induce both $CD8^+$ and $CD4^+$ T cells. Vaccination with MHC class I/II-loaded DCs has been shown to both increase the frequency of tumor-specific $CD8^+$ T cells and co-activate $CD4^+$ T cells, thereby further improving clinical responses (4, 23).

Recently, the first commercial DC vaccine, Sipuleucel-T, was approved by the FDA for the treatment of prostate cancer. In a phase III clinical trial, Sipuleucel-T showed an increase of 4.3 months in median survival and 33% reduction in the risk of death (24). Nevertheless, despite the significant benefit in median survival, satisfying clinical effects in terms of solid anti-tumor immune responses were only observed in a minority of patients, strongly suggesting that further optimization is warranted (25). Other trials also underscore the potential of DC vaccination in metastatic cancers, especially in melanoma. In this setting, it was shown that autologous DCs loaded with tumor antigens are safe and capable of inducing tumor antigen-specific immune responses in a substantial part of the vaccinated patients (26). Despite these growing successes, DC vaccination has not yet proven to be a method superior to other protective immunity stimulating vaccine strategies (27, 28). Anti-tumor responses are hampered by the tumor microenvironment which seems to be very immunosuppressive, especially in patients with a high tumor load (20, 29).

IMMUNOSUPPRESSIVE TUMOR MICROENVIRONMENT

Although DC vaccination succeeds in activating the immune system, resulting in the presence of tumor-specific T cells, the clinical success of these treatments is still limited. The lack of clinical efficacy can be mostly attributed to an immunosuppressive tumor microenvironment, which is very successful in attenuating T cell-mediated responses. The tumor microenvironment consists of tumor cells, fibroblasts, endothelial cells, and infiltrating immune cells together with extracellular matrix components. Infiltrating immune cells can be either beneficial or detrimental depending on the nature of the infiltrating cells. The presence of tumor-infiltrating lymphocytes (TILs) has been associated with improved survival of patients with prostate, breast, colorectal, ovarian cancer, or melanoma (30–33). On the other hand, the presence of Tregs or myeloid-derived suppressor cells (MDSCs), which can inhibit anti-tumor immune responses, is associated with decreased survival (34–36). Furthermore, tumor cells express a number of proteins on their cell surface capable of inactivating tumor-specific T cells, as detailed below. Therefore, immunotherapy strategies aimed at inducing T cell-mediated anti-tumor immunity need to include an approach to break tolerance to the tumor.

INHIBITORY CHECKPOINT RECEPTORS AND LIGANDS

T cell functions, both priming and effector, can be attenuated by inhibitory checkpoint receptors and ligands expressed by T cells themselves, DCs and other immune cells, or tumor cells. The most important co-inhibitory checkpoint receptors are CTLA-4 and PD-1, in combination with the PD-1 ligands, PD-L1 (B7–H1), and PD-L2 (B7-DC), all belonging to the B7 receptor superfamily. Other B7 family members, such as B7–H3 and B7–H4, and the unrelated receptors herpes virus entry mediator (HVEM), inhibitory receptor Ig-like transcript-3 and -4 (ILT3 and 4), T cell immunoglobulin mucin protein-3 (TIM-3), and lymphocyte activation gene-3 (LAG-3) are also involved in inhibiting T cell function (2) (**Figure 2**).

Cytotoxic T lymphocyte-associated antigen-4

Cytotoxic T lymphocyte-associated antigen-4 is a homolog of the co-stimulatory molecule CD28 and it is exclusively expressed on $CD4^+$ and $CD8^+$ T cells after activation. Tregs represent an exception, as they constitutively express CTLA-4. In contrast, CD28 is constitutively expressed on all T cell subsets regardless of activation (37–39). CD28 and CTLA-4 are closely related in structure and both bind to the ligands CD80 and 86 present on APCs, such as DCs, macrophages, and B cells (10). Although the expression

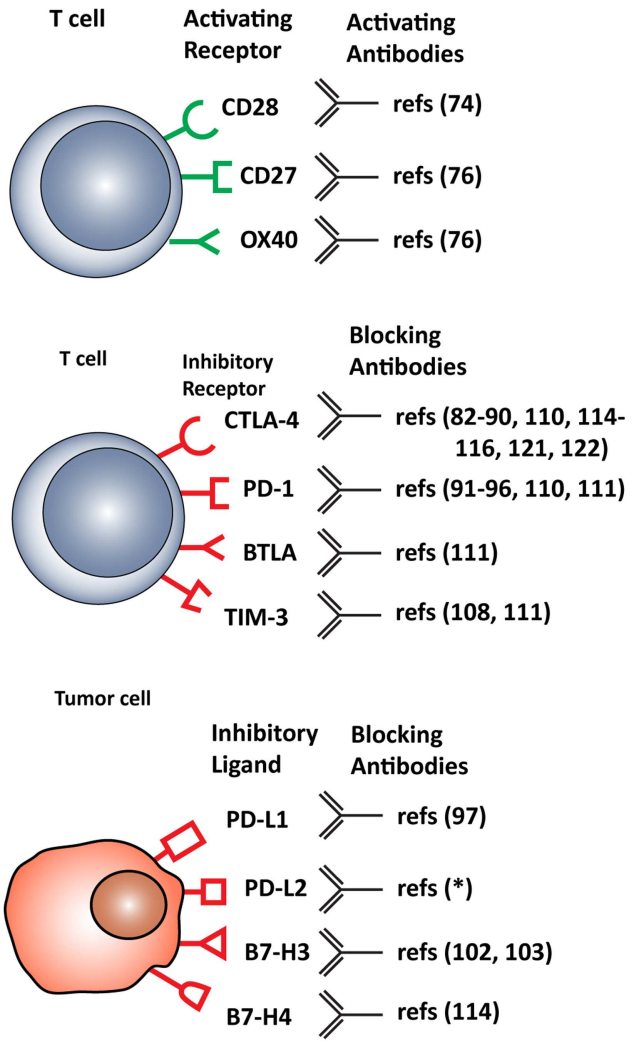

FIGURE 2 | Stimulatory and inhibitory molecules expressed in the tumor microenvironment targeted for therapeutic intervention. Schematic visualization of stimulatory and inhibitory receptors expressed on T cells and on various tumor cells. Specific monoclonal antibodies are in development that either function as agonists, enhancing T cell activation, or antagonist, blocking T cell inhibitory molecules. *(ClinicalTrials.gov identifier: NCT00658892).

of CTLA-4 on the cell surface is low compared to CD28, it has a higher affinity for the ligands (37, 40). CTLA-4 receptor ligation leads to inhibition of T cell proliferation, cell cycle progression, and IL-2 synthesis (41, 42). Its cell surface expression is induced by CD28 ligation, implying that it serves as an internal checkpoint, downregulating CD28 stimulation and thereby attenuating immune responses (43). Despite its apparent role in attenuating T cell activation, CTLA-4 seems to be required for effective anti-tumor immunity, as this molecule also affects T cell polarization. *In vivo* studies have shown that CTLA-4-deficiency in mice causes severe lymphoproliferative disorders, promoting a Th2 phenotype (44) while a Th1 phenotype is required for efficient anti-tumor immunity (45, 46).

PD-1/PD-L1 and PD-L2

Another inhibitory member of the B7 receptor family is PD-1. This receptor is more widely expressed than CTLA-4, being found on $CD4^+$ and $CD8^+$ T cells (including Treg cells), B cells, monocytes, and at lower levels on NK cells (47, 48). Its major function is limiting autoimmunity and T cell activity in peripheral tissues in response to infection (49, 50). Tumor cells can exploit these characteristics by inducing expression of PD-1 on tumor-specific T cells, thus suppressing their effector function and eventually leading to T cell exhaustion and immune resistance in the tumor microenvironment (51, 52). Two ligands are known to interact with PD-1: PD-L1 (53) and PD-L2 (54). PD-L1 is expressed on resting and activated T cells, B cells, DCs, mast cells, macrophages, endothelial cells, tumor cells, and other cells within the tumor microenvironment (55–57). This tumor-associated PD-L1 expression was reported to increase apoptosis of infiltrating T cell (52, 58). Interestingly, PD-L1 does not only interact with the PD-1 receptor, but also with CD80 expressed on T cells, inhibiting T cell activation, and cytokine production (59). PD-L2 has a higher affinity for PD-1 than PD-L1, and although its expression was thought to be restricted to APCs, it has been shown to be expressed by normal and cancer-associated fibroblasts, a specific subset of B cells, activated T cells and tumor cells (60). PD-L1 expression on tumor cells is associated with aggressive tumor behavior, poor prognosis, and elevated risk of death, while for PD-L2 such correlations were not significant (60, 61).

B7–H3 and B7–H4

Two additional B7 family co-inhibitory ligands are B7–H3 and B7–H4. The receptors for these molecules have not been identified yet, but they are expected to be expressed by activated T cells (62). B7–H3 is constitutively expressed on a wide variety of tissues, and its expression on leukocytes is dependent on inflammatory cytokine stimulation (63). In contrast, expression of B7–H4 is more restricted, being found on T cells, B cells, monocytes, and DCs after activation (64). Many human cancers express B7–H3 and B7–H4, which is generally associated with poor prognosis (65, 66). Furthermore, B7–H3 seems to be upregulated on endothelial cells of the tumor vasculature and on tumor-associated macrophages (TAMs) (67).

Other co-inhibitory molecules

Other co-inhibitory receptors, which can be exploited by tumors to dampen anti-tumor immune responses, are HVEM, ILT3 and 4, TIM-3, and LAG-3. HVEM is expressed by immature DCs and interacts with its ligands "B and T lymphocyte attenuator" (BTLA), LIGHT, and CD160, all expressed on T cells (68). HVEM interaction with BTLA inhibits T cell responses, promotes T cell survival, and mediates Treg suppression (2). BTLA and CD160 compete for the same cysteine rich domain of HVEM with a similar affinity, but a different dissociation rate, suggesting a dominant inhibitory role for CD160 (69). It seems that HVEM ligation of BTLA inhibits immune responses against tumor cells, while LIGHT exerts pleiotropic effects to increase this response (70).

Ig-like transcript-3 and -4 are inhibitory receptors both expressed by monocytes, macrophages, and DCs (71, 72). The corresponding ILT3 ligand is not yet known, but since ILT3 can

directly suppress T lymphocyte function, it is likely to be expressed on T cells (73, 74). In several cancers, ILT3 has been found to mediate the immune escape mechanism by impairing T cell responses (75). Furthermore, ILT4-expressing DCs block efficient CTL differentiation, a mechanism that is used by tumors, which upregulate ILT4 to evade the immune system (76).

T cell immunoglobulin mucin protein-3 is a checkpoint receptor expressed by IFN-γ-secreting $CD4^+$ T helper and $CD8^+$ cytotoxic T cells. When interacting with its ligand, galectin-9, it triggers cell death and terminates immune responses driven by these T cells. The most important role of TIM-3 in anti-tumor immunity involves T cell exhaustion and stimulation of MDSC-mediated suppression of T cell responses (77).

Lymphocyte activation gene-3, a CD4 homolog, is an activation-induced cell surface molecule that binds with high affinity to MHC class II on APCs. LAG-3 is expressed by T cells, NK cells, B cells, and plasmacytoid DCs. By binding to its ligand, it inhibits T cell expansion and controls the size of the memory T cell pool (78). When upregulated on Tregs, LAG-3 can modulate suppressive Treg function (79). Furthermore, LAG-3 plays important role in both the homeostatic maintenance and activation-induced expansion of DCs (80). Co-expression of LAG-3 and PD-1 on tumor-infiltrating $CD8^+$ T cells, induced by either tumor-derived APCs or cytokines secreted in the tumor microenvironment, contribute to the establishment, and maintenance of an immunosuppressive tumor microenvironment (81).

Taken together, these data show that tumors have evolved intriguing mechanisms to exploit the balance between co-stimulation and co-inhibition by skewing this toward co-inhibition and thus dampening anti-tumor immune responses. In fact, this has become a crucial aspect of immunosuppression in the tumor microenvironment, effective against both natural and induced anti-tumor immunity.

CLINICAL INTERVENTION

Strategies to break or neutralize the aforementioned inhibitory mechanisms present in the tumor microenvironment are currently being developed. This can be accomplished by either decreasing activity of suppressive molecules or by increasing activity of stimulatory molecules. Monoclonal antibodies are being produced that bind to co-stimulatory/co-inhibitory receptors and their ligands, and thereby either antagonizing those that suppress immune responses or activating others that amplify immune responses. A number of these are now being tested in the clinic (**Figure 2**).

TARGETING CO-STIMULATOR MOLECULES WITH AGONISTIC ANTIBODIES

As the effector T cells in the tumor microenvironment seem to be immunosuppressed, a logical step would be to develop antibodies that can (re)activate T cell responses in the microenvironment. In this setting, the most attractive target seems to be the co-stimulatory molecule CD28. Agonistic antibodies targeting CD28 were developed and entered clinical testing. However, a trial in which an agonist anti-CD28 monoclonal antibody (TGN1412) was tested has since become a cautionary tale to the power of the immune system. This antibody led to an unexpected release of cytokines (cytokine storm) in the volunteers, causing severe toxicities (82). This incident highlighted the potential dangers of agonistic antibodies and severely decreased the interest in further developing these strategies for many years. Recently, this interest has been re-kindled and a number of agonistic antibodies are being explored. In particular, members of the tumor necrosis factor receptor (TNF-R) family have emerged as targets for enhancing tumor-specific responses. This includes CD27, GITR, 4-1BB, CD30, and OX-40, which are expressed on tumor-specific T cells, and antibodies targeting these molecules are under investigation in several (pre)clinical studies (83). Among these, only anti-4-1BB, and anti-CD30 antibodies had success in clinical trials as monotherapies. A phase II study with anti-4-1BB treatment showed promising results, but was eventually terminated due to, unexpectedly high, grade 4 hepatitis (84). A recent phase III study of anti-CD30, brentuximab vedotin, as treatment of relapsed patients with Hodgkin lymphoma resulted in a 71% objective response rate (85). CD40, another TNF-R family member, which is expressed on APCs, muscle cells, fibroblasts, and basophils is also being explored as a potential target for immunotherapy (86). Several phase II trials for the treatment of myeloma and diffuse large cell lymphoma are currently testing the efficacy of the humanized anti-CD40 antibody dacetuzumab (10). Also, a new, fully human, anti-CD40 monoclonal antibody was evaluated in a phase I trial and considered safe for further clinical development (87). So, the development of agonistic antibodies is in progress, but the question remains if such indiscriminate activation of T cells will lead to efficient anti-tumor immune responses, or whether the risk of severe adverse effects or autoimmune activation will prove to be too high.

TARGETING CO-INHIBITORY MOLECULES WITH ANTAGONISTIC ANTIBODIES (BLOCKADE OF IMMUNE CHECKPOINTS)

CTLA-4 blockade

Just like agonistic antibodies might lead to non-specific activation of the immune system and cause more harm than good, so too was blockade of CTLA-4 questioned initially (**Figure 3**). Most CTLA-4 expressing T cells are not tumor-specific and *ctla-4* KO mice exhibited a lethal autoimmune and hyperimmune phenotype, predicting immune toxicity in human CTLA-4 blockade (88, 89). However, when CTLA was only partially blocked with antibodies, severe toxicity was prevented and significant anti-tumor responses were observed in mice (90). These pre-clinical results led to the development of two, fully human, anti-CTLA-4 monoclonal antibodies for the treatment of several cancers, including melanoma and renal cell carcinoma (10): ipilimumab, an IgG1 antibody with plasma half-life of 12–14 days (Bristol–Myers Squibb) and tremelimumab, an IgG2 antibody with a plasma half-life of 22 days (Pfizer).

Ipilimumab was tested in a phase II trial but failed to reach its endpoint of tumor regression. Regardless, it was still tested in a large phase III trial and became the first drug to demonstrate survival benefit in patients with advanced melanoma in a randomized trial. Metastatic melanoma patients were treated with ipilimumab, with or without a glycoprotein 100 (gp100) peptide vaccine, or with gp100 alone (91). Patients treated with ipilimumab, with or without gp100, had a 3.5-month survival benefit compared to the group treated with gp100 alone (91). In a second randomized

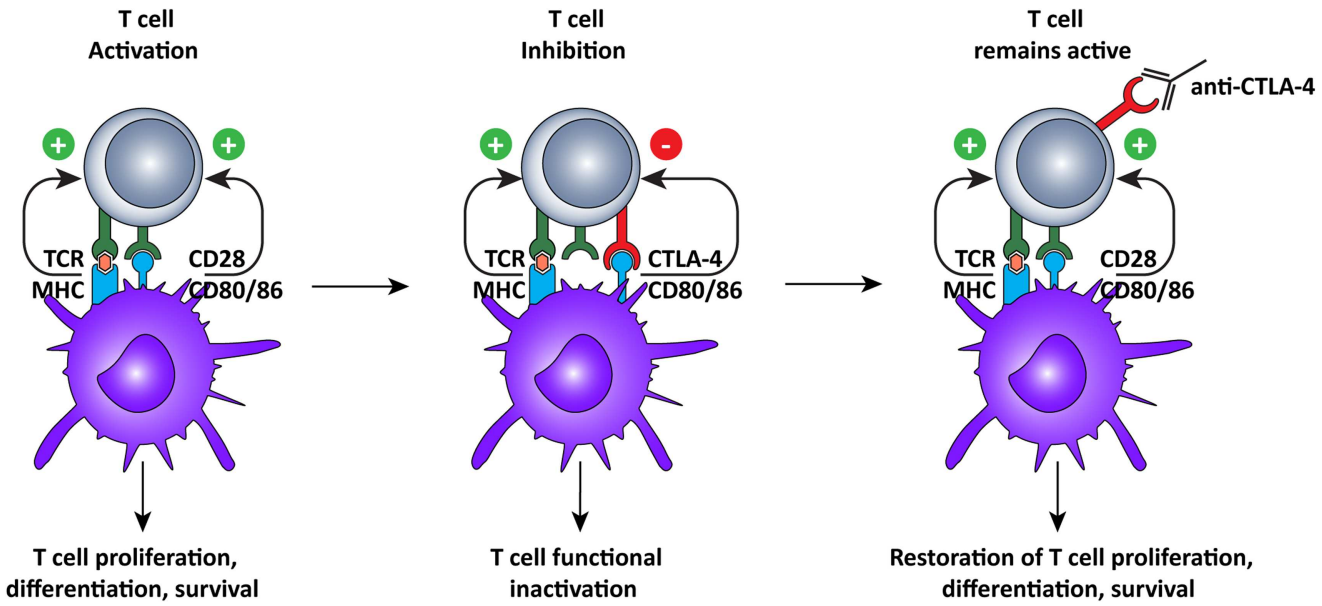

FIGURE 3 | Cytotoxic T lymphocyte-associated antigen-4 blockade restores T cell activation. After recognition of MHC:peptide complex by the TCR, the second signal for T cell activation is provided by binding of CD80 or 86 to CD28 on the T cells. This interaction leads to cell surface expression of CTLA-4, which has a higher affinity for CD80/86, thus interrupting the activation signal. Additionally, the signal delivered via CTLA-4 down-regulates T cell function and inhibits excessive expansion of activated T cells. Anti-CTLA-4 monoclonal antibodies bind to CTLA-4, and block the interaction with CD28, which is again free to interact with CD80/86, prolonging T cell activation and amplifying T cell-mediated immunity against tumors.

trial, the combination of ipilimumab with standard dacarbazine treatment showed an increase in overall survival of 2.1 months compared to dacarbazine alone (11.2 vs. 9.1 months). Additionally, there was an increase in patients with at least 3 years survival (20.8 vs. 12.2%) (92). In contrast, tremelimumab did not show any significant improvement in survival of patients with metastatic melanoma when tested in a phase III trial in comparison with standard chemotherapy. As a result the development program was abruptly terminated (93).

Although showing promising results, the use of CTLA-4 blockade still presents many challenges for the clinic. There is a significant rate of adverse reaction caused by the treatment, with up to one third of the patients experiencing immune-related serious adverse effects (irSAEs) up to grade 3 or 4, ranging from dermatitis to severe chronic colitis or acute hepatitis (94–96). Furthermore, the efficacy of CTLA-4 blockade as a single treatment seems to be limited to intrinsically immunogenic tumors such as melanoma (97, 98).

PD-1 pathway blockade

In contrast to CTLA-4 blockade, PD-1 blockade was expected to be less toxic, based on the different phenotype associated to PD-1 knockout mice. Whereas *ctla-4* KO mice died from a lethal lymphoproliferative disorder at a very young age, some colonies of *pd-1* KO mice lived over a year before expressing lupus-like symptoms (49, 88).

The first fully human anti-PD-1 IgG4 antibody, nivolumab (MDX1106) was tested in a phase I clinical trial. The trial was conducted on patients with different solid tumors and showed promising results, as it was relatively well tolerated (14% grade 3–4 irSAE) and showed anti-tumor activity (99). Long-term follow-up on three patients that participated in the phase I trial (melanoma, renal cell carcinoma, and colorectal cancer) showed the presence of memory T cells that mediated a persistent anti-tumor immune response in the absence of continued therapy, indicating long-term clinical benefit of PD-1 blockade (100). A subsequent, dose-escalating, phase I trial, conducted in melanoma patients, also showed that nivolumab was well tolerated. Immune-related toxicities were mild, less frequent (21% grade 3–4 irSAE), and less severe than those observed with ipilimumab (101–103). This antibody is now being tested as first-line treatment in a phase III trial compared to dacarbazine for treatment of metastatic melanoma (ClinicalTrials.gov identifier: NCT01721772). Two other anti-PD-1 antibodies were tested in clinical trials: lambrolizumab (MK3475) and pidilizumab (CT-011). Lambrolizumab was shown to have a response rate of 38% in patients with melanoma, and induced a durable progression-free survival rate of longer than 7 months and low grade toxic effects (104). Pidilizumab was tested in hematopoietic malignancies, where anti-tumor activity was observed in one patient with follicular lymphoma and one with acute myelogenous leukemia (105). These results seem to indicate that PD-1 blockade, like CTLA-4 blockade, can overcome immunosuppressive mechanisms present in the tumor microenvironment and reactivate pre-existing tumor-specific T cells.

The ligands of PD-1, PD-L1, and PD-L2, which are expressed on both tumor and normal cells within the tumor microenvironment (55–57, 60), are also interesting targets for immunotherapy (**Figure 4**). A recent clinical trial of the anti-PD-L1 antibody,

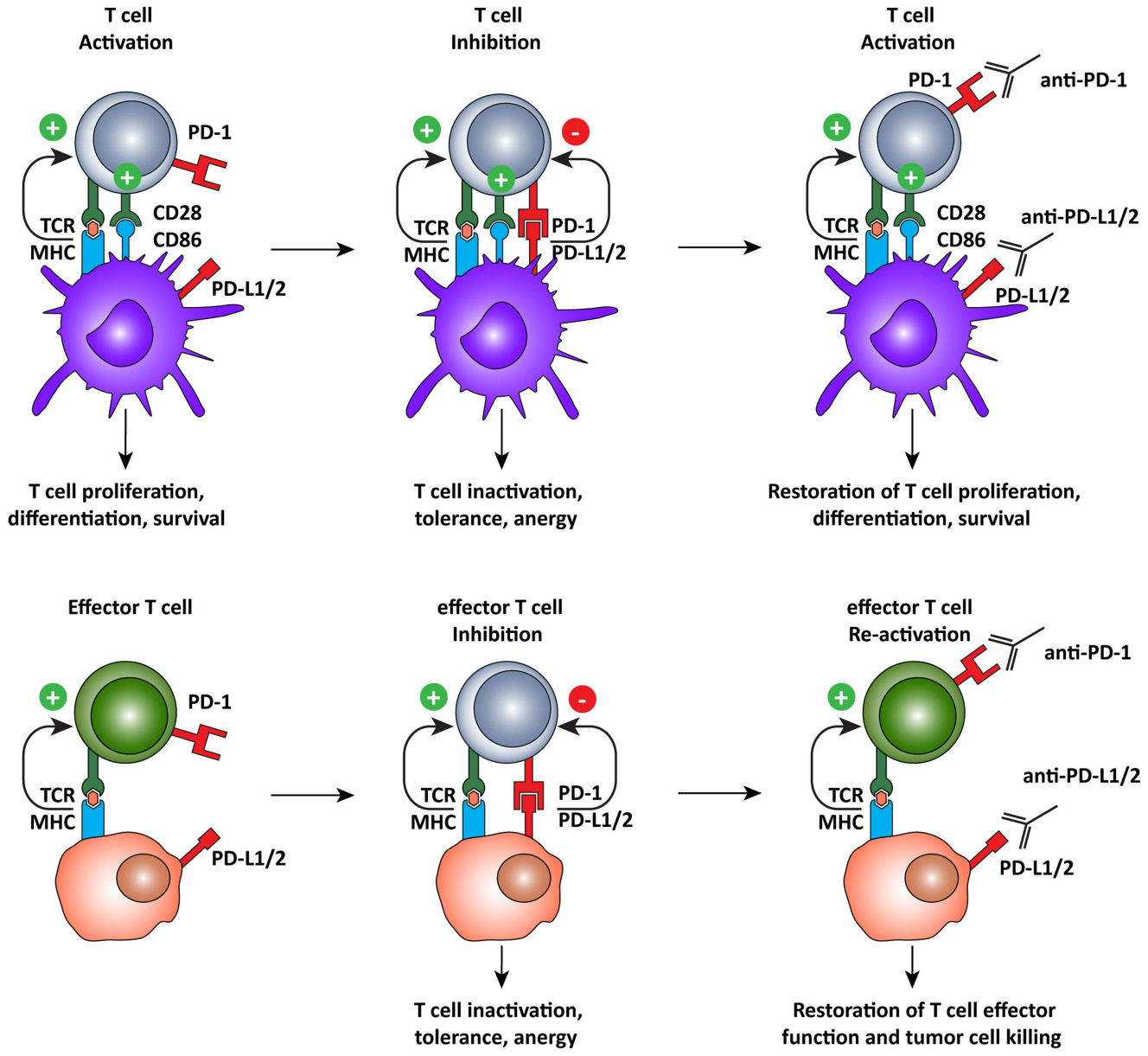

FIGURE 4 | Programed cell death-1 pathway blockade promotes tumor-specific T cell activation and elimination of tumor cells. The PD-1 pathway operates on two different levels, regulating both T cell activation by DCs and the effector function of antigen-specific T cells. PD-1 pathway blockade by monoclonal antibodies directed against PD-1, or its ligands, promotes T cell activation by shifting the balance of signals delivered by the DC from suppressive to activating. In the tumor microenvironment, tumor-specific T cells recognize tumor cells but are subsequently inactivated by the expression of PD-L1 or PD-L2 on the tumor cell, inducing tolerance and anergy. When rescued, by blocking the PD-1 pathway, T cells recognize antigen in the periphery and, in the absence of PD-1 engagement, they assume full effector function and eliminate tumor cells.

BMS-936559, showed durable tumor regression and prolonged stabilization of the disease, with only 9% of patients experiencing grade 3 or 4 irSAE (106). PD-L2 blockade is currently being evaluated in a clinical trial but results are not yet available (ClinicalTrials.gov identifier: NCT00658892). Nonetheless, it appears that targeting PD-L1 and PD-L2 may be a strategy to limit off-target toxicity, while still combating the immunosuppressive tumor microenvironment.

B7–H3/H4 blockade

Both B7–H3 and B7–H4 receptors are expressed in tumors of prostate, non-small-cell lung, pancreatic, gastric, and skin cancer (107). In a non-small-cell lung cancer study, high B7–H3 or B7–H4 expression correlated with lymph node metastasis (108). In spite of being expressed on tumor cells, the role of B7–H3 as an inhibitory molecule is still not clear. Some studies have shown that expression of B7–H3 on tumor cells or tumor vasculature is associated with

an increased risk of death (109), while others have shown that B7–H3 expression is associated with prolonged patient survival and TIL infiltration (110). In mouse models, B7–H3 overexpression on tumor cells was shown to favor tumor regression (107). However, it has also been reported that antagonistic antibodies could enhance *in vitro* T cell proliferation (111). Altogether, the uncertainty on the exact function of B7–H3 makes its implication in cancer therapy rather difficult. Notwithstanding, a B7–H3 targeting antibody has been developed, which mediates potent cellular toxicity against a broad range of tumor cell types, and is currently being tested in a clinical trial (112). B7–H4 seems to have a clearer role in inhibiting T cell functions (113), and *in vitro* models have shown that antibody-mediated blockade of B7–H4 could restore anti-tumor T cell responses, making it an interesting target for clinical application (114).

Blockade of other immune checkpoints

Up till now, clinical intervention strategies have focused primarily on the B7 family, as highlighted above; other immune checkpoint pathways are not as well established and therefore research has been limited to pre-clinical, *in vitro* studies or mouse models. Nonetheless, these may prove to be important therapeutic targets in the future. The interaction of HVEM with several ligands, such as BTLA, CD160, and LIGHT, makes the balance between co-stimulatory and co-inhibitory signals rather complex. It also seems that signaling is bidirectional, depending on the specific combination of interactions. Therefore, immune checkpoint blockade in this pathway is not as straightforward as with other molecules (68, 96). Further delineation of the complex HVEM/BTLA/CD160/LIGHT pathway is required to elucidate the possibilities in immune blockade therapies.

The inhibitory receptors ILT3 and 4 also play an important role in the regulation of the immune response. In patients with melanoma, and carcinomas of the colon, rectum, and pancreas, ILT3 was reported to mediate immune escape mechanism, resulting in largely unsuccessful immune therapies (75). Soluble ILT3 protein induces differentiation of $CD8^+$ T cell and impairs T cell responses (75, 115). This could be restored by anti-ILT3 antibody or depletion of the soluble ILT3 from the serum. Thus, blocking ILT3 may prove to be an important adjuvant in immunotherapy. ITL4 upregulation on DCs was reported to cause blockade of cytotoxic T cell differentiation (76). Blockade of this receptor would therefore also be useful to augment DC function and enhance immune responses to cancer.

On the other hand, blockade of TIM-3 seems more feasible, as anti-TIM-3 displayed modest prophylactic and therapeutic activity against a small fraction of sarcomas in a mouse model. Furthermore, IFN-γ production from $CD8^+$ cells, but not from $CD4^+$ cells, was shown to be critical for the anti-tumor effect of the anti-TIM-3 treatment (116). TIM-3 blockade seems to mainly stimulate anti-tumor responses via NK cell-dependent mechanisms, while blockade of another family member, TIM-4, induces $CD8^+$ cytotoxic T cells (117).

COMBINATORIAL IMMUNOTHERAPIES

Up till now, immune checkpoint blockade has mostly been developed as monotherapy with marginal efficacy, but the use of these immune checkpoint blockades in combinatorial regimens might improve clinical efficacy. Although these therapies could be combined with the usual suspects, radio- and chemo-therapy, the most benefit might reside in the combination with other immunotherapeutic approaches. However, extra care is warranted, as manipulation of the tightly controlled balance of immune activation vs. inhibition could be dangerous.

COMBING IMMUNE CHECKPOINT BLOCKADES

As CTLA-4 blockade and PD-1 pathway blockade target different mechanisms of T cell inactivation, there is a rational for expecting synergy when combining both these immune checkpoint blockades. Taking the high prevalence of irSAEs associated with these treatments when used as monotherapy into account, combining them is a risky proposition at best. Nevertheless, this combination treatment (anti-CTLA-4 mAb, ipilimumab and anti-PD-1 mAb, nivolumab) was tested in a recent, dose-escalating, phase I trial, and the results were very promising. The highest dose showed a 53% objective response and all patients had at least 80% tumor shrinkage. As might be expected, immune toxicity was higher than with monotherapy but this was a small increase compared to the increase in clinical response (118). Although the patient numbers in this trial were small, there was clear synergistic effect when combining these two immune checkpoint blockades. This is currently being confirmed in a phase III trial.

Programed cell death-1 pathway blockade in combination with other co-inhibitory molecules has also proven to be potentially useful. Blockade of the HVEM ligand, BTLA, in combination with PD-1 and TIM-3 blockades enhanced IL-2-producing $CD8^+$ T cell expansion in an *in vitro* melanoma model (119). Also, when anti-PD-1 and anti-TIM-3 antibodies are combined, a significant decrease of tumor size was found, compared to PD-1 blockade alone (99). Since LAG-3 and PD-1 are co-expressed on $CD4^+$ and $CD8^+$ T cells, several combinatorial therapies have been explored. Frequency and effector function of $CD8^+$ T cells were increased after LAG-3 and PD-1 blockade in a mouse model of epithelial ovarian cancer (81). Additionally, another *in vivo* study, applying a dual anti-LAG-3/anti-PD-1 antibody therapy showed a markedly improvement of the overall condition of mice challenged with tumor, that were resistant to single antibody treatment (120).

COMBINING CTLA-4 BLOCKADE WITH DC VACCINATION

The main problems encountered with anti-CTLA-4 treatment are the resistance of advanced tumors, due to a strong tumor-induced T cell tolerance, which may be partially PD-1 pathway mediated, and a lack of tumor specificity (121). Thus, a novel and potentially successful strategy would be the combination of DC vaccination with CTLA-4 blockade. This is supported by several pre-clinical tumor models, showing that CTLA-4 blockade on its own is not very potent in triggering a specific anti-tumor response, but when combined with agents that prime immune responses, such as DC vaccination, it might become very effective. In a study using a EL4 lymphoma mouse model, the administration of a single dose DC vaccination in combination with anti-CTLA-4 monoclonal antibody resulted in the rejection or retarded tumor growth in 60% of the challenged tumor mice, while either the vaccine or CTLA-4 blockade were ineffective when administered alone (122).

The combination of CTLA-4 blockade and vaccination with B16 or SM1 cells, genetically modified to express GM-CSF, showed enhanced efficacy and tumor regression when administered in a B16 melanoma model and SM1 mammary carcinoma model, respectively. In the same experimental set up, monotherapy was again ineffective (123, 124). Taken together, these data suggest that CTLA-4 blockade in combination with DC vaccination could break tolerance to tumor-specific antigens, resulting in tumor clearance, and long-term host immunity after tumor re-challenge.

COMBINING PD-1 PATHWAY BLOCKADE WITH DC VACCINATION

In parallel with therapies which combine CTLA-4 blockade with DC vaccination, strategies for interfering with PD-1 pathway to enhance DC vaccination are being explored in pre-clinical studies. Administration of poly(I:C), a TLR3 agonist, as a tumor vaccine adjuvant was shown to selectively upregulate PD-L1 expression on mouse $CD8\alpha^+$ DCs. Although the $CD8\alpha^+$ DCs were able to promote cross-priming of $CD8^+$ T cells, there was a lack of expansion of the primed tumor antigen-specific $CD8^+$ T cells. This resulted in a failure to establish an anti-tumor immune response, suggesting that TLR3-induced PD-L1 expression on DCs may act as a negative regulator of $CD8^+$ T cells expansion. Thus, blockade of PD-L1 on poly(I:C)-activated DCs might improve the anti-tumor efficacy of DC-based vaccines (125). In fact, in a B16 murine melanoma model treated with tumor peptide-pulsed DCs, concurrent systemic administration of anti-PD-L1 antibody resulted in a higher number of melanoma peptide-specific $CD8^+$ T cells. Surprisingly, in spite of the increased number of tumor-specific T cells, there was no significant reduction in tumor growth (126). Additionally, blockade of PD-1/PD-L1 immune checkpoint in a murine breast cancer model was shown to effectively augment DC function in the stimulation of tumor-specific T cell mediated cytotoxicity, leading to efficient induce anti-tumor immunity (127). Together, these studies support blocking of the PD-1 pathway as a means to enhance the efficacy of DC vaccination.

COMBINING CTLA-4 BLOCKADE WITH OTHER CANCER TREATMENTS

Combining CTLA-4 blockade with other immunotherapeutic approaches or targeted therapies is also proven to be beneficial in several mouse models and has also entered clinical trials. The combination of the GM-CSF-engineered allogeneic vaccine GVAX with ipilimumab showed an improved overall survival of 29.2 months in patients with metastatic castration-resistant prostate cancer, but also displayed increased toxic effects when compared to therapy with either agent alone (128). In a recent phase I trial, ipilimumab was combined with the BRAF inhibitor vemurafenib in melanoma patients with the V600E BRAF mutation. However, the study was closed due to unforeseen hepatotoxicity, again highlighting the need for extreme care when combining these treatment modalities (129). In a long-term study, patients with metastatic melanoma treated with ipilimumab and IL-2 showed a 17% complete response rate, which is promising but still needs to be verified in a randomized trial (130).

DISCUSSION AND FUTURE PERSPECTIVE

Although cancer immunotherapy development is now flourishing and recognized as a novel important treatment modality by oncologists, it had a rough start, as most immunotherapeutic agents were not effective in early trials (131). Over the years, the field of immunotherapy has evolved and matured. Growing knowledge about the immunosuppressive tumor microenvironment has provided some new promising checkpoint targets, as described above. This has all resulted in FDA-approved treatment modalities such as ipilimumab and Sipuleucel-T. Notably, the introduction of ipilimumab to the clinic has provided a boost to cancer immunotherapy, particularly keeping in mind that ipilimumab is the first anti-cancer treatment approved that does not target the tumor but rather targets the immune system. However, despite having clear therapeutic benefits and showing the possibility of long-term survival, there are still some challenges ahead. The first problem is the observed spectrum of toxicity or irSAEs, causing inflammatory and autoimmune reactions. This was to be expected on the basis of the pre-clinical mouse models, but is nonetheless a serious problem. In clinical trials, up to 25–30% of patients treated with ipilimumab suffer from grade 3 to 4 SAEs, including dermatitis, colitis, and hypophysitis (94). Unfortunately, there is no correlation between anti-tumor effect and the severity of these side effects, meaning that the patients experiencing these irSAE do not necessarily benefit from an anti-tumor effect. In this regard, blockade of PD-1 or PD-L1 has proven to be a much milder treatment alternative. In theory, blockade of CTLA-4 seems to be more effective than PD-1 pathway blockade, as it might lead to the activation or induction of new tumor-specific T cells, in addition to (re)activation of pre-existing tumor-specific T cells. However, both CTLA-4 and PD-1 pathway blockade seem to have similar clinical efficacy, but PD-1 pathway blockade is reported to have significantly fewer instances of irSAE.

A second drawback of immune checkpoint blockade is the lack of specificity. These treatment modalities are designed to "release the brakes" on the immune system, leading to indiscriminate immune activation, which is the cause of the irSAEs. This also means that only patients that already have pre-existing, naturally induced, tumor-specific T cells, which are being suppressed by these immune checkpoints, will benefit. Although CTLA-4 blockade is thought to be able to activate new tumor-specific T cells, this has never been proven in humans, and up till now this therapy seems to be the most effective in immunogenic tumors. Furthermore, a recent study has shown that patients whose tumors had higher expression of genes involved in immune function before the start of the treatment responded better to ipilimumab. Furthermore, expression of genes associated with T cell responses were increased after ipilimumab therapy. These findings support the concept that ipilimumab may be more efficacious in subjects who have pre-existing natural, albeit ineffective, anti-tumor immune responses (97).

Combining non-toxic DC vaccination with immune checkpoint blockade might be a good combination, exploiting the advantage of DC vaccination: the induction of tumor-specific T cells to compensate for the lack of specificity in checkpoint blockade. Conversely, this combination might also compensate for the lack of potency of the DC-induced tumor-specific T cells, by blocking the expression of inhibitory molecules in the tumor microenvironment (**Figure 5**). A recent phase II trial, assessing safety and dosage, showed that the combination of DC vaccination

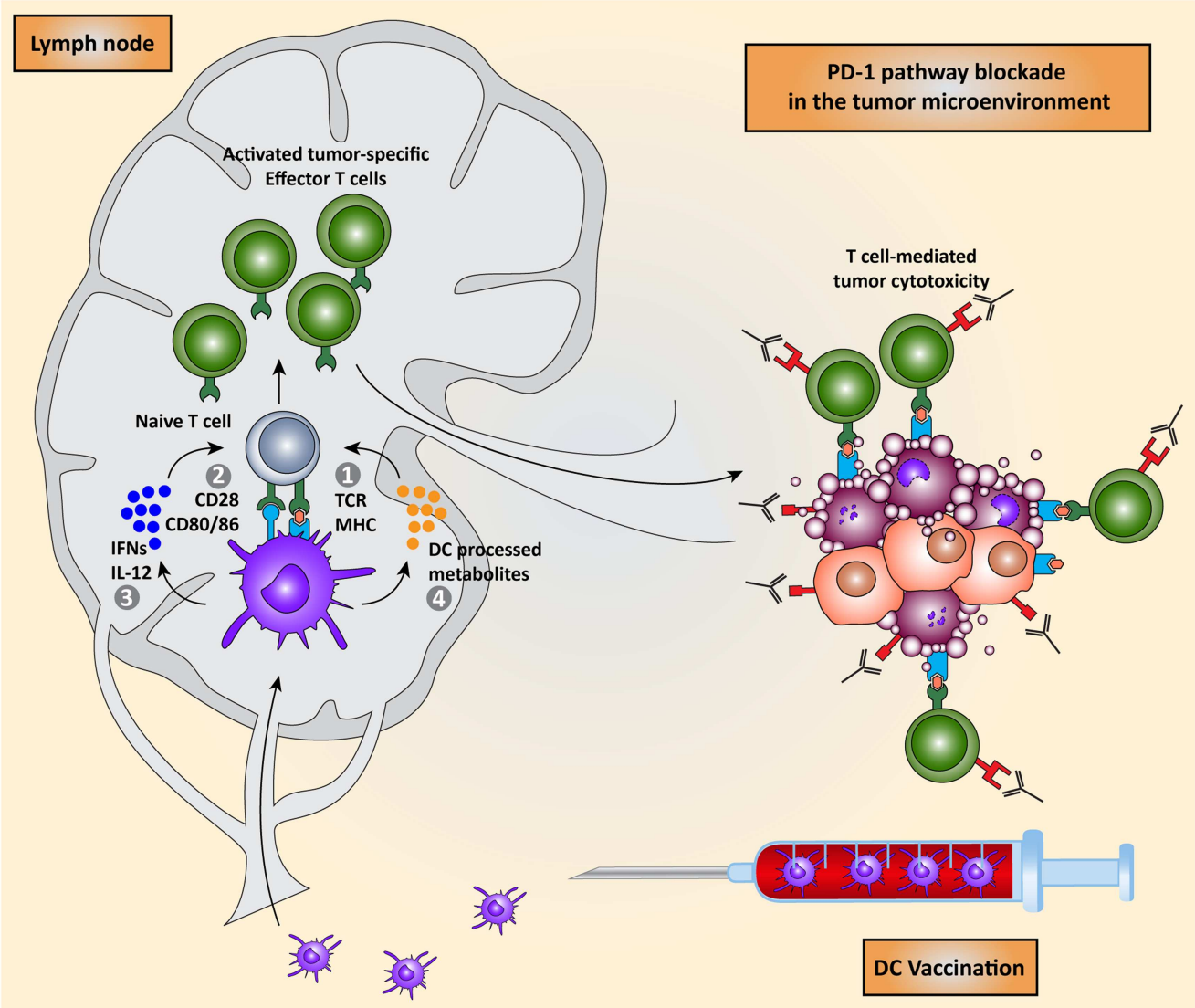

FIGURE 5 | Combining DC vaccination with immune checkpoint blockade. DC vaccination of cancer patients leads to the induction of tumor-specific T cells that migrate to the tumor microenvironment. PD-1 pathway blockade synergistically potentiates the effects of DC vaccination by blocking PD-1/PD-L1 induced immunosuppression leading to enhanced tumor cell killing.

with dose escalation of the CTLA-4 blocking antibody, tremelimumab, resulted in objective and durable tumor regressions, while irSAE were limited to grade 3 (132, 133). This indicates that this combination regiment in practice does not lead to extra toxicity compared to CTLA-4 blockade and might be even less toxic. Although not directly compared, or in combination with DC vaccination, recent results in clinical trials indicate that PD-1 pathway blockade are more active and less toxic than CTLA-4 blockade. This might be due to the more tumor-specific mode of immune activation. Additionally, PD-1 blockade might also provide the possibility of using biomarkers to select patients that will respond. In the nivolumab trial, 9 out of 25 patients with PD-L1 expression in the tumor responded to treatment while none of the 17 patients whose tumor did not express PD-L1 responded. Additionally, a recent study identified increased PD-1 expression on tumor-specific $CD8^+$ T cells in melanoma patients, indicating that the PD-1 pathway is actively contributing to suppressing immune response in melanoma patients. Together, these results warrant for a phase I/II trial combining DC vaccination with PD-1 pathway blockade where patients are selected for increased PD-1 expression on $CD8^+$ T cells or expression of PD-L1 by their tumor (103, 134).

Finally, it should be mentioned that also other options exist to combat inhibitory molecule expression within the tumor microenvironment. Recent studies have indicated that chemotherapeutic drugs can potentiate the immune system via the so-designated "off-target effects" (135). For example, platinum-based chemotherapeutics were shown to downregulate PD-L1 on DCs

while also downregulating PD-L2 on both DCs and tumor cells. This resulted in enhanced T cell activation and increased tumor cell recognition (136, 137). Chemotherapy may therefore also potentiate the effect of immunotherapy by improving DC maturation and function and eliminating suppressive cells (138).

In summary, cell-based immunotherapeutic approaches, such as DC vaccination, are promising strategies for cancer treatment. After years of optimization, these therapies are succeeding in inducing tumor-specific T cells in cancer patients. Unfortunately, so far this was insufficient to produce clear clinical benefits, albeit long-lasting responses were seen in a small proportion of the patients. A major factor hampering these novel therapies is the immunosuppressive tumor microenvironment. When migrating to the tumor site, tumor-specific T cells end up in an environment specialized in suppressing anti-tumor immune responses. Tumor cells accomplish this in large part by exploiting immune checkpoints, designed to dampen immune responses after infection and prevent autoimmunity. Recent antibody-based immunotherapeutic approaches, specifically designed to block these T cell inhibitory pathways, facilitate effector T cells to attack the tumor. The main drawback of checkpoint blockade antibodies is their lack of specificity, especially since it is not possible to determine in advance if tumor-specific T cells are present.

In this review, we highlighted the crucial role of the intricate regulatory molecular networks governing T cell activation and effector function, immune checkpoints, in the context of anti-tumor immunity and how these mechanisms are hijacked by tumors in order to suppress immune responses. More importantly, we discussed the use of immune checkpoint blockades as cancer treatment and provided a rationale for combining these with DC vaccination as a potentially superior alternative to blocking multiple immune checkpoints. Altogether, our growing knowledge about the immunosuppressive tumor microenvironment, and especially how it can be manipulated in a therapeutic setting, has opened up a fantastic opportunity to synergistically combine checkpoint blockade, especially PD-1 pathway blockade, with DC vaccination or adoptive T cell transfer. This will result in a powerful combination regiment leading to tumor clearance and immunological memory, which can mediate long-lasting tumor regression.

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Dendritic cell-targeted approaches to modulate immune dysfunction in the tumor microenvironment

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There has been enormous progress this past decade in the understanding of the biology of dendritic cells (DCs) along with increasing attention for the development of novel dendritic cell (DC)-based cancer therapies. However, the clinical impact of DC-based vaccines remains to be established. This limited success could be explained by suboptimal conditions for generating potent immunostimulatory DCs as well as immune suppression mediated by the tumor microenvironment (TME). Therefore, strategies that optimize the potency of DC vaccines along with newly described therapies that target the TME in order to overcome immune dysfunction may provide durable tumor-specific immunity. These novel interventions hold the most promise for successful cancer immunotherapies.

Keywords: cancer, immunotherapy, dendritic cells, tumor microenvironment, immune checkpoints

INTRODUCTION

Naturally occurring anti-tumor immune responses in cancer patients and in murine tumor models are commonly impaired. Tumor escape as a result of immuno-editing or through local effects of the tumor microenvironment (TME) disables many components of the immune response and ultimately limits the success of immunotherapy. Suppression or modulation of tumor-associated dendritic cell (DC) function by the TME is thought to play a major role in impairing the development of potent anti-tumor immune responses and promoting tumor progression. This review provides an overview of the mechanisms by which the tumor cells and tumor-associated cells co-opt many endogenous host factors and physiological pathways in order to impair immunogenic DC function. An updated overview of DC-based tumor immunotherapies and strategies to target the TME in order to overcome DC dysfunction and treat cancer patients will be discussed. Understanding the underlying mechanisms involved in the modulation of DC-based anti-tumor immunity by the TME will provide opportunities for improving the efficacy of cancer immune therapies.

DENDRITIC CELL BIOLOGY

The 2011 Nobel Prize in Medicine or Physiology was awarded to Ralph Steinman for his discovery of dendritic cells (DCs) and their role in adaptive immunity. DCs are the most potent professional antigen-presenting cells (APCs), able to activate adaptive immunity through their capacity to sample the environment and capture, process, and present antigens to T cells (1). Immature DCs in peripheral tissues can capture antigens but due to absence of co-stimulatory molecules, antigen presentation results in induction of tolerance through T-cell deletion, anergy and induction of regulatory, or suppressor T cells. Exposure to pathogens, however, engages the process of maturation which guarantees a well-controlled and targeted immune response.

While maturing, DCs lose their ability to capture antigen, and acquire new features such as enhanced antigen processing and presentation (through upregulation of surface MHC-II molecules); enhanced ability to migrate (through upregulation of the chemokine receptor CCR7); and increased capacity to stimulate T and B cells through cytokine secretion and co-stimulatory molecules. DCs uptake antigens through different mechanisms (phagocytosis, macropinocytosis, and endocytosis) and process them into peptides that are loaded on MHC molecules. The peptide/MHC complexes are then presented to naïve T cells in the lymphoid tissues. Binding of T cells to the MHC-antigen complex and co-stimulatory molecules on DC surface (CD80, CD86, CD40) results in the activation and subsequent differentiation of T cells into effector cells endowed with unique functions and cytokine profiles, capable of launching an antigen specific response. Extracellular antigens (bacteria, parasites, toxins) are presented onto MHC-II molecules and presented to $CD4^+$ T cells whereas intracellular antigens (viral proteins) are presented on MHC-I molecules to $CD8^+$ T cells. Importantly, DCs are the only APCs able to present extracellular antigens onto MHC-I molecules to $CD8^+$ T cells, a process called cross-presentation that is crucial for anti-tumor immunity, however, not all DC subsets may be capable of efficient cross-presentation, and the degree to which they do may be dependent upon the nature of the antigen and route of delivery. Myeloid DCs (mDCs, also know as classical or conventional DCs) and plasmacytoid DCs (pDCs) are the two main subsets of DCs. mDCs are key players in immune responses against pathogenic organisms and tumors. They differentiate from myeloid progenitors, express CD11c and include the dermal DCs, Langerhans cells, interstitial DCs, and interdigitating DCs. mDCs are found in peripheral tissues, lymphoid organs, and in the blood and secrete large amounts of IL-12 upon activation. IL-12 mediates enhancement of the cytotoxic activity of NK cells and $CD8^+$ cytotoxic T lymphocytes, is involved in the differentiation of naive

T cells into T_H1 cells, and stimulates the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) by T and NK cells cells. Blood mDCs includes BDCA1$^+$ (CD1c$^+$) and BDCA3$^+$ (CD141$^+$) DCs. Recent studies have identified BDCA3$^+$ (CD141$^{+)}$ DCs as the human counterpart of CD8α$^+$ murine DCs that share several phenotypic and functional properties such as their expression of TLR3 and their ability to secrete IL-12 and IFN-β. Although BDCA3$^+$ DCs are widely thought to cross-present antigens more efficiently than other DC populations, new findings show that DC populations may be comparably effective at presenting exogenous antigens to CD8$^+$ T cells as long as the antigen is delivered to early endocytic compartments (2, 3).

Plasmacytoid DCs are the principal producers of type-I interferons (IFNs) in response to microbial and viral infection. They express CD123, BDCA2, and BDCA4 and are primarily found in blood and lymphoid organs such as the thymus, bone marrow, spleen, tonsils, and lymph nodes under steady state conditions. pDCs infiltrate various type of tumor but their role in anti-tumor immune responses remains to be defined as some reports suggest they can promote tumor growth (4).

Dendritic cell maturation involves the production of cytokines that play a role in CD4$^+$ T-cell polarization into T_H1, T_H2, and T_H17. Differentiation of T_H1 cells, key players in immune responses against intracellular pathogens, tumors, and viruses, is driven by IL-12-mediated secretion. Development of T_H2 cells, involved in responses against parasites (but detrimental in the setting of anti-tumor responses), is though to be induced by the lack of IL-12 as well as by IL-4, thymic stromal lymphopoietin (TSLP), and Matrix metalloproteinase 2 (MMP-2). TGF-β, IL-1β, IL-6, and IL-23 have been implicated in T_H17 polarization. DCs can also induce naïve CD4$^+$ T cells to differentiate into T follicular helper cells whose function is to help B cells to differentiate into antibody-secreting cells, as well as into regulatory T cells which function is to suppress immune responses. DCs also play a role in CD8$^+$ T-cell differentiation into effector cytotoxic T lymphocytes. In addition to their ability to mediate adaptive immunity, DCs activate innate immune responses, such as NK cells' cytotoxicity and cytokine production trough their secretion of IL-12, IL-18, and type I-IFN. DCs also activate γδ T cells, another essential component of the anti-tumor immune response. Finally, DCs are also thought to play a role in the induction of effector memory T cells (TEM) that differentiate into central memory T-cell (TCM), but the mechanisms involved are still unclear. Altogether, these findings make DCs the ideal candidate for cancer immunotherapy as they activate overall immune responses.

Interestingly, it has been shown recently that in early stages of tumor progression, DCs are immunocompetent and able to induce the expansion of specific T-cell responses, whereas DCs in advanced tumors become immunosuppressive (5). Understanding the underlying mechanisms involved in the modulation of DC-based anti-tumor immunity by the TME will provide opportunities for improving the efficacy of immune therapies.

TUMOR MICROENVIRONMENT: A HOT BED OF IMMUNO-SUPPRESSIVE ACTIVITY

Despite the induction of tumor-specific T-cell responses in many patients, DC vaccines have not translated into durable therapeutic responses. Indeed, the TME employs several mechanisms that inhibit DCs to induce efficient anti-tumor responses (**Figure 1**).

Immuno-suppressive molecules

Several tumor-derived factors such as IDO/TDO, CCL-2, VEGF, TGF-β, M-CSF, GM-CSF, IL-6, and IL-10 have been reported to negatively impact DC functions. TGF-β results in impairment of DC function and accumulation/differentiation of Tregs, myeloid-derived suppressor cells (MDSC), and detrimental M2 macrophages (6). IL-6 and M-CSF switch differentiation from monocytes to macrophages rather than DCs (7). IL-10 is able to convert immunostimulatory DCs into tolerogenic APCs and induce anergic cytotoxic CD8$^+$ T cells (8). We and others found that inhibition of MAPK pathway in human BRAF^{V600E} mutant melanoma lines reduced production of immuno-suppressive cytokines (IL-6, IL-10, VEGF) and restored IL-12 and TNF-α production by DCs (9, 10). Stat3 is another signaling pathway that has emerged as a critical regulator of immuno-suppressive cytokines. An excellent review discusses various signaling pathways activated in cancers such as Stats, MAPK, and β-catenin (11). The chemokine CCL2 recruits inflammatory monocytes which express its receptor CCR2, as well as metastasis-associated macrophages, therefore promoting malignancy (12). VEGF is involved in several mechanisms of tumor pathophysiology such as inhibition of DC differentiation (13). Several monoclonal antibodies have been developed against VEGF or its receptor in order to prevent angiogenesis and have shown clinical benefits in various cancers. Activation of antigen-specific-Tregs for potent suppressor activity has been shown to be achieved by pDCs and cDC through secretion of the enzyme indoleamine 2,3-dioxygenase (IDO) (14, 15).

Regulatory T cells

CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs play a crucial role in maintaining a suppressive environment and inhibiting anti-tumor responses. Tregs express the inhibitory receptors CTLA-4, PD-1, and Tim-3 which contribute to their suppressive function through different mechanisms (16). Some studies indicate that Tregs through CTLA-4 can induce the down regulation of the co-stimulatory molecules CD80 and CD86 on DCs (17). Moreover, Tregs compete for the cytokine IL-2 with other immune cells through their expression of its receptor CD25 with a 100-fold higher affinity (18). Similar mechanisms might apply for other cytokines such as IL-7, IL-15, and IL-12. Finally, Tregs can secrete two of the main immuno-suppressive cytokines: IL-10 and TGF-β that blunt anti-tumor effector cells such as CD4$^+$, CD8$^+$, and NK.

Immuno-suppressive myeloid cells

It is well established that subpopulations of myeloid cells are critical mediators of tumor initiation, angiogenesis and metastasis and are able to inhibit anti-tumor immune responses through a variety of mechanisms. MDSCs for instance play a crucial role in immune evasion within tumors through several immuno-suppressive mechanisms that blunt effector T-cell responses (19). They suppress CD8$^+$ T-cell anti-tumor immunity (20, 21) and induce the differentiation of Tregs (22). Not only do they secrete immuno-suppressive cytokines such as IL-10 but also express high levels of NOS (nitric oxide synthase) involved in T-cell apoptosis (19, 23), and Arginase-1 which impair the local proliferative

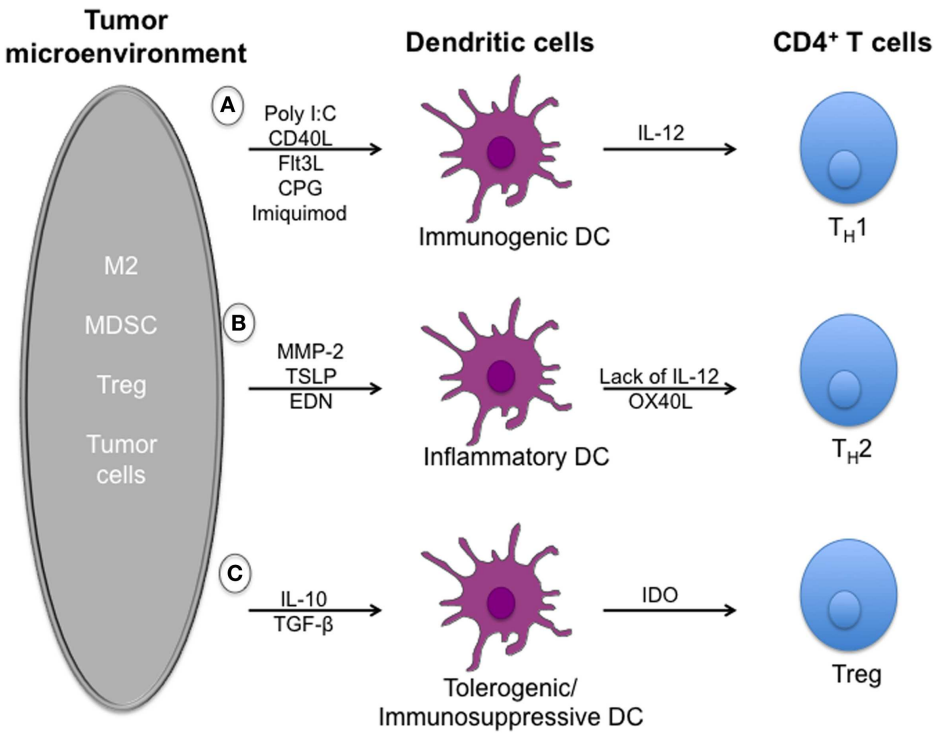

FIGURE 1 | Dysregulation of dendritic cell-mediated anti-tumor immune responses by tumor microenvironment. Effector T cells can recognize and kill tumor targets after activation by immunogenic dendritic cells. However, a number of soluble mediators, including TGFβ, IL-10, and alarmins, that are secreted by immuno-suppressive cells such as Treg cells, MDSCs, and tumor cells can dysregulate dendritic cells function and limit T-cell effector functions. **(A)** Exposure to pathogens induces the maturation of immunogenic dendritic cells that secrete large amounts of IL-12 upon activation. IL-12 mediates enhancement of the cytotoxic activity of NK cells and $CD8^+$ cytotoxic T lymphocytes, is involved in the differentiation of naive T cells into T_H1 cells, and stimulates the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) from T and NK cells cells. **(B)** In the tumor microenvironment, development of detrimental/suboptimal T_H2 cells is induced by alarmins such as TSLP, EDN, and MMP-2 through mechanisms depending on inflammatory DCs. **(C)** Immuno-suppressive cytokines such as IL-10 and TGF-β are responsible for the induction of immature/tolerogenic/immuno-suppressive DCs able to promote the accumulation of regulatory T cells. Tregs play a crucial role in maintaining a suppressive environment and inhibiting anti-tumor responses.

capacity of T cells (24). Macrophages have also been shown to facilitate tumor growth. In the context of TME, macrophages are skewed toward an M2-altered functional phenotype able to produce lower levels of pro-inflammatory cytokines (IL-1β, TNF-α, IL-12) and higher levels of immuno-suppressive cytokines such as IL-10, TGF-β, and VEGF (25–27). Immunotherapeutic approaches aimed at skewing detrimental M2 macrophages into an immuno-competent M1 phenotype may promote effective anti-tumor immunity.

Induction of T_H2 cells through the expression of alarmins

Alarmins are naturally occurring endogenous mediators, rapidly released in response to infection and/or tissue injury by several cell types. These "danger signals" function to alert the host immune system of cell and tissue trauma through activation and recruitment of effector cells of innate and adaptive immunity (28). DCs are able to sense alarmins present in the TME through surface and intracellular receptors.

Matrix metalloproteinase 2 is expressed by cancer and/or stromal cells and is associated with later tumor stages, increased dissemination, and poorer prognosis/survival (29, 30). We have shown that MMP-2 can directly modulate innate and adaptive immune responses toward melanoma by not only being recognized by specific $CD4^+$ and $CD8^+$ tumor-infiltrating T cells, but also by modulating DC function to polarize T_H2 responses. We recently identified two pathways whereby MMP-2 functions as a human endogenous "conditioner" that skews $CD4^+$ T cells toward a detrimental T_H2 phenotype. MMP-2 degrades the type I IFN receptor (IFNAR1), thereby preventing STAT1 phosphorylation necessary for IL-12 production (31). Furthermore, we identified that MMP-2 is a direct ligand for TLR2 on DCs, and found that their interaction leads to OX40L up-regulation and T_H2 skewing (Godefroy et al., in revision).

Thymic Stromal Lymphopoietin has also been described to modulate DC function and drive T_H2 responses (32). TSLP produced by tumor cells has been shown to induce detrimental T_H2 cells responsible for increasing tumor growth in breast cancer and pancreatic cancer through the secretion of IL-13 and IL-4 (33, 34).

These findings support the idea that blocking antibodies for MMP-2/TLR2 or TSLP/TSLPR interactions represent a promising strategy for cancer therapy through their ability to polarize type-1 immune responses.

Another alarmin, Eosinophil-derived neurotoxin (EDN) has been shown to activate the TLR2–MyD88 signal pathway in DCs and enhances T_H2 immune responses (35).

Inhibition of antigen presentation by alteration of MHC molecules and loss of tumor antigen expression

The TME alters the ability of DCs to effectively present antigen due to a down regulation or loss of MHC molecules and genes associated with antigen presentation such as transporter associated with antigen processing (TAP), low-molecular-weight protein (LMP), and β2-microtubulin (36). Another mechanism of tumor escape is the loss of tumor-associated antigens (TAA): the natural selection of tumor subclones poorly recognized by the immune system which can thereby survive immune pressure (37).

Expression of inhibitory ligands

Immune checkpoints such as CTLA-4, PD-1, Tim-3, LAG3, ICOSL, GITRl, and B7H3 are inhibitory receptors that regulate immune responses to insure tolerance and prevent auto-immune diseases. They will be discussed in section "Therapies Targeting TME." CD47, a ligand for SIRPα, is a "don't eat me" signal for phagocytic cells, whose function is to block phagocytosis. CD47 overexpression by human solid tumor cells represents another mechanism of tumor escape by preventing tumor cells to be phagocytosed and eliminated (38). Recent data has shown that its blockade by neutralizing antibodies inhibits migration and metastasis in a variety of tumor models.

Study of the TME is critical to better understand how tumors harness surrounding cells to escape immunity and support their growth. This combined with a better understanding of DC biology should lead to the development of new strategies that effectively restore DC activity and induce tumor detection and the generation of potent anti-tumor responses.

DENDRITIC CELL-BASED TUMOR IMMUNOTHERAPIES

The immune system can eradicate tumors as shown by spontaneous regression of primary and metastatic melanoma (39) and regression of tumors after adoptive transfer of T cells (40). The potential for DCs to launch adaptive immunity makes them ideal candidates for cancer immunotherapy (**Figure 2**). However this approach alone does not overcome TME-induced DC dysregulation. Therefore, targeting TME may improve the clinical benefit of DC-based vaccines.

DC-BASED VACCINES

Ex vivo-generated DCs pulsed with antigens

The clinical impact of DC immunotherapy has been limited despite the induction of tumor-specific T-cell responses in many patients and occasional tumor regressions. At this point, the first and only cell-based cancer vaccine approved by the FDA is Provenge® from Dendreon. Provenge is an autologous antigen-pulsed DC-based cancer vaccine for patients with metastatic prostate cancer based on the results of a phase III randomized trial that demonstrated a more than 4-month median improvement in overall survival compared with a placebo vaccine. Overall, clinical trials have demonstrated the feasibility and safety of DC vaccines in phase I and II but have failed to demonstrate strong efficacy in large phase III trials (41, 42).

Many reasons may explain this lack of success with DC vaccines. There is currently no consensus on the optimal strategy to generate

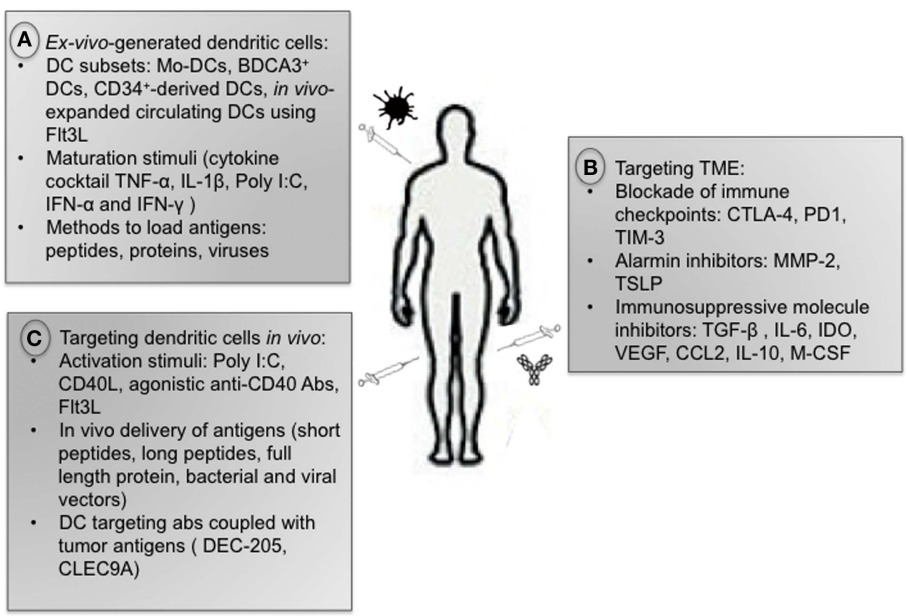

FIGURE 2 | Anti-tumor immunotherapies. **(A)** There is currently no consensus on the optimal strategy to generate DCs for immunotherapeutic use regarding DC subsets, maturation stimuli, and methods to load antigens. **(B)** Therapies aiming at reprograming the immuno-suppressive TME are very promising, including blockade of immune checkpoints as well as inhibitors of alarmins and immunosuppressive cytokines. **(C)** Strategies targeting DCs *in vivo* include administration of activation stimuli (Poly I:C, CD40L, Flt3L), *in vivo* delivery of tumor antigens, and administration of tumor antigens coupled with antibody against DC surface receptors.

DCs for immunotherapeutic use. DC-based immunotherapies require optimization at several levels: the maturation stimuli used, the type and form of antigen to be administered, the subset and the number of DCs to inject, and the frequency, route, and site of the injection. Studies in humans and mice have emphasized that different DC subsets are endowed with specialized functions, and a good vaccine should utilize these subsets in a coordinated way. Questions remain as to whether the classical *ex vivo*-generated moDCs widely used in immunotherapy are the most effective means of inducing clinically significant anti-tumor immunity. Some studies use DCs derived from $CD34^+$ precursors (43) or *in vivo*-expanded circulating DCs using Flt3L. Recent findings provide the basis for a new approach relying on $BDCA3^+$ DCs as anti-tumor vaccines, as they seem to be a key subset for cross-presentation of cell-associated antigens (44). Further characterization of these DCs will enable rational approaches to target them to improve vaccine efficacy. Looking forward, the main challenge for using $BDCA3^+$ DCs will be to develop an efficient way to generate them in large numbers. Alternative vaccination strategies such as the delivery of tumor antigens *in vivo* to $BDCA3^+$ DC subsets using antibodies specific to cell surface receptors such as CLEC9A has been proposed. However, more recent findings previously discussed (2, 3) suggest that this approach may not offer an inherent advantage and that the optimal strategy would be to target antigens to early endosomes. This approach would not only increase cross-presentation by $BDCA3^+$ DCs but also extend cross-presentation to more abundant DC subsets therefore maximizing $CD8^+$ T-cell responses *in vivo*. It is worth pointing out that Dendreon uses circulating blood DCs as the adjuvant, not the commonly used moDCs. A large study directly comparing all DC subsets side by side for their capacity to induce CTL and T_H1 responses after activation with various stimuli is warranted.

Another critical parameter to induce DC-mediated potent anti-tumor responses is the choice of DC maturation stimuli. Indeed, proper DC maturation prior to vaccination is necessary to prevent induction of tolerance through Tregs. To mature DCs, some clinical trials have used a standardized cocktail of pro-inflammatory cytokines composed of TNF-α, IL-1β, IL-6, and PGE2 that was shown to induce up-regulation of MHC molecules, co-stimulatory molecules as well as CCR7 (45). However, other findings have suggested that DC matured with this cytokine cocktail were not optimal as they fail to induce IL-12p70 production and may induce Treg and T_H2 cells (46–48). A novel cytokine cocktail consisting of TNF-α, IL-1β, Poly I:C, IFN-α, and IFNγ has shown good results including DC-mediated IL-12 secretion (49, 50). Alternative maturation strategies *via* direct administration of immune activators such as TLR agonists, Flt3L, or CD40L has been shown to improve DC function *in vivo* (51). Several TLR ligands are currently being tested in clinical trials including LPS (TLR4), CpG (TLR9), Poly I:C (TLR3), Imiquimod (TLR7), and Resiquimod (TLR7 and TLR8).

Another factor that may explain the limited success of DC-based vaccines is the less-than optimal migration of DC vaccines to secondary lymphoid organs. Studies showed that most of the injected DCs remain at the site of injection, <5% reaching the draining lymph nodes (52). Administration of DCs via multiple routes or directly into the lymph nodes may improve DC migration and clinical responses.

Finally, it's worth mentioning that most of the clinical trials treat patients with late stage cancers, whereas the most suitable stage for cancer vaccine is likely to be early disease when tumor volume is low.

In vivo delivery of antigens (non-targeted vaccines)

Contrary to previous assumptions, we showed that DC vaccines have an insignificant role in directly priming $CD8^+$ T cells, but instead function primarily as vehicles for transferring antigens to endogenous APCs, which are responsible for the subsequent activation of T cells (53). This finding highlights the need to develop strategies directly targeting endogenous DCs. Moreover, *in vivo* targeting of DCs represents a more economical option for DC immunotherapy as it bypasses the expensive and labor-extensive *ex vivo* DC generation process described previously.

Tumors express several well-characterized antigens that are recognized by the immune system. TAA can be antigens derived from oncogenic viruses (human papilloma virus E6 and E7 proteins), the products of mutations, differentiation antigens (tyrosinase, TRP-1, TRP-2, gp100, Melan A/MART1), overexpressed variants (Her2/neu), or self-antigens specifically upregulated on tumors. Strategies that target antigen presentation on both MHC-I and II molecules are ideal as both $CD4^+$ and $CD8^+$ T cells are required to launch potent protective anti-tumor immune response. Immunotherapies using short peptides from tumor antigens present limitations because they can only be used in patients with known HLA alleles that present these epitopes in the absence of natural processing. Alternatively, full-length protein vaccines often suffer from lack of consistent $CD8^+$ T-cell induction, likely due to inefficient cross-presentation of the exogenous antigen by DCs. In contrast, synthetic long peptides are efficiently presented to both $CD4^+$ and $CD8^+$ T cells by DCs as well as non-professional APCs (54). The use of bacterial and viral vectors represents another alternative for loading tumor antigens on DCs. Genes encoding TAAs are inserted into the vector while gene encoding virulence of replication factors are deleted out. In some case, the vector may encode for cytokines and co-stimulatory molecules and therefore induce maturation of DCs, thereby bypassing the need for a separate maturation stimuli (55). The disadvantage of the method is that pre-existing immunity against the bacteria or virus vector may reduce their ability to induce immune responses.

Antigens coupled with DC surface antigens (in vivo targeting of DCs)

Endogenous DCs can be targeted to either deliver tumor-associated-antigens and/or to provide co-stimulatory signals. Candidates for the targeting of DC-specific molecules include Fc receptors, CD40, and C-type lectin receptors such as DEC-205, DC-SIGN, CLEC9A, mannose receptor, and Dectin-1. TAAs can be directly delivered *in vivo* using chimeric proteins composed of an antibody that is specific for the DC receptor fused to a selected antigen or to long peptides. Specific targeting of antigens to DCs *in vivo* has been shown to elicit potent $CD4^+$ T-cell responses as well as an enhancement of antibody responses (56–58). $CD8^+$ T-cell responses are less efficiently induced, unless boosted in a "prime" fashion such as with pox vectors (59). To avoid the induction of antigen-specific tolerance, this strategy requires DC activation

signals. Most of the studies are performed in mice and further investigations are needed to determine the efficacy in humans and to identify the best candidate to target.

Optimizing DC vaccines is necessary but to be successful, immunotherapeutic approaches also need to overcome TME-induced immune suppression to be able to potentiate the efficacy of DC vaccines *in vivo* and translate to overall improved clinical outcomes.

THERAPIES TARGETING TME

Among the most promising approaches to activating therapeutic anti-tumor immunity is the blockade of immune checkpoints. Among checkpoint molecules, CTLA-4 blockade was the first shown to enhance anti-tumor responses (58). CTLA-4 is an homolog of CD28 whose binding to its ligands CD80 and CD86 induces an inhibitory signals to CTLA-4-expressing T cells. CTLA-4 blockade using neutralizing antibodies (Ipilimumab and Tremelimumab) targets both effector and regulatory Tregs and has been shown to enhance immune responses and show promising clinical responses in melanoma patients (60). Ipilimumab (Yervoy) has recently been approved by the FDA for the treatment of unresectable or metastatic melanoma, based on improved overall survival in treated patients (61). Anti-CTLA-4 treatment is currently being tested for other cancers.

PD-L1, a ligand for the exhaustion marker PD-1, is expressed by different TME-infiltrating cell types including DCs. Blockade of PD-L1 induced durable tumor regression and prolonged stabilization of disease in patients with advanced cancers, including non-small-cell lung cancer, melanoma, and renal-cell cancer (62). Moreover, clinical trials using an anti-PD-1 antibody (nivolumab) reported promising results in patients with advanced cancer (63). Nivolumab is now in phase III testing. Interestingly, early results presented at the ASCO 2013 meeting suggested higher response rates to PD-1 pathway blockade in patients whose tumors express PD-L1, while combinatorial blockade of CTLA-4 and PD-1 increased anti-tumor immunity when compared to blocking either single checkpoint alone, although toxicity was higher (Grosso, abstract #3016; Callahan, abstract #9012).

Similarly to CTLA-4 and PD-1, Tim-3 belongs to the group of immune checkpoints and is a potential therapeutic target. Although there is no clinical data yet, Tim-3 has been reported to be co-expressed with PD-1 on human tumor-specific $CD8^+$ T cells, and dual blockade of both molecules significantly enhances the *in vitro* proliferation and cytokine production of human T cells (64–66). *In vivo* studies have shown that Tim-3 blockade alone, or in combination with PD-1 blockade, is able to control tumor growth in four different tumor models, including melanoma (66, 67). Moreover, recent findings have shown that tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1 (68) therefore defining a new mechanism whereby the TME suppresses anti-tumor immunity. We found that NK cells from melanoma patients were dysfunctional/exhausted and that Tim-3 blockade was able to reverse this exhausted phenotype and improve NK cell function. Altogether, those findings suggest that Tim-3 blockade would improve anti-tumor immunity by not only targeting T cells, but also DCs and NK cells.

CONCLUSION

Dendritic cells have the potential to initiate specific anti-tumor immune responses, but several components of TME can modify their phenotype and function to transform immuno-competent DCs into immuno-suppressive DCs. The TME not only abrogates specific T-cell response but also induces DCs to exert immuno-suppressive and pro-angiogenic functions. Thus, combinatorial approaches that (1) reprogram the immuno-suppressive TME; (2) improve DC function; and (3) enhance T-cell immunity, should provide durable tumor-specific immunity and hold the most promise for successful immune-base cancer therapies.

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Dendritic cell-based immunotherapy for myeloid leukemias

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Acute and chronic myeloid leukemia (AML, CML) are hematologic malignancies arising from oncogene-transformed hematopoietic stem/progenitor cells known as leukemia stem cells (LSCs). LSCs are selectively resistant to various forms of therapy including irradiation or cytotoxic drugs. The introduction of tyrosine kinase inhibitors has dramatically improved disease outcome in patients with CML. For AML, however, prognosis is still quite dismal. Standard treatments have been established more than 20 years ago with only limited advances ever since. Durable remission is achieved in less than 30% of patients. Minimal residual disease (MRD), reflected by the persistence of LSCs below the detection limit by conventional methods, causes a high rate of disease relapses. Therefore, the ultimate goal in the treatment of myeloid leukemia must be the eradication of LSCs. Active immunotherapy, aiming at the generation of leukemia-specific cytotoxic T cells (CTLs), may represent a powerful approach to target LSCs in the MRD situation. To fully activate CTLs, leukemia antigens have to be successfully captured, processed, and presented by mature dendritic cells (DCs). Myeloid progenitors are a prominent source of DCs under homeostatic conditions, and it is now well established that LSCs and leukemic blasts can give rise to "malignant" DCs. These leukemia-derived DCs can express leukemia antigens and may either induce anti-leukemic T cell responses or favor tolerance to the leukemia, depending on co-stimulatory or -inhibitory molecules and cytokines. This review will concentrate on the role of DCs in myeloid leukemia immunotherapy with a special focus on their generation, application, and function and how they could be improved in order to generate highly effective and specific anti-leukemic CTL responses. In addition, we discuss how DC-based immunotherapy may be successfully integrated into current treatment strategies to promote remission and potentially cure myeloid leukemias.

Keywords: dendritic cells, immunotherapy, active, myeloid leukemia, minimal residual disease, leukemia stem cells

INTRODUCTION

During the last century our molecular and mechanistic understanding of the immune system and the immunosurveillance of solid and hematological tumors has advanced extensively. For hematological tumors especially, the demonstration of the graft-vs.-leukemia (GvL) effect of allogeneic hematopoietic stem cell transplantation (aHSCT) and donor lymphocyte infusions (DLIs), as well as the discovery of leukemia-associated antigens (LAAs) was of fundamental importance in order to translate, implement, and optimize immunotherapies against myeloid leukemias. Consequently, active and passive immunotherapy approaches, such as peptide- and dendritic cell (DC)-based vaccines using LAAs, monoclonal antibodies (mAbs), and the *in vitro*-generation of leukemia-specific cytotoxic T cells (CTLs) for adoptive transfer have recently yielded promising results in pre-clinical models and clinical trials (1–4). To maximize their efficacy, these immunotherapies have to be implemented into the treatment strategy in conjunction with standard treatments of care for each patient individually. Here, we summarize recent advances in DC-based active vaccination using LAAs and discuss this method as an attractive supplementary immunotherapeutic strategy in the context of current standard treatments for myeloid leukemias.

CLASSIFICATION, EPIDEMIOLOGY, AND CLINICAL MANIFESTATIONS OF CML AND AML

Hematologic malignancies are neoplasms of the blood-forming system. Conceptually, these neoplasms can be divided into four different subsets (myeloid, lymphoid, mixed myelo-lymphoid, and histiocytic/dendritic neoplasms, see **Figure 1**) (5, 6). Myeloid neoplasms can be further grouped into acute myeloid leukemia (AML) and chronic myeloid disorders depending on the percentage of bone marrow (BM) infiltration by immature blasts. 20% and more infiltrating immature blasts define the cut-off criterion for AML. Chronic myeloid disorders such as chronic myeloid leukemia (CML) bear the risk of evolving into AML. Experimental studies revealed that myeloid leukemias in general are of clonal origin, suggesting genesis from a single leukemia stem cell [LSC, reviewed in Ref. (7)].

Chronic myeloid leukemia is caused by translocation of chromosomes 9 and 22 in a hematopoietic stem cell (HSC) resulting

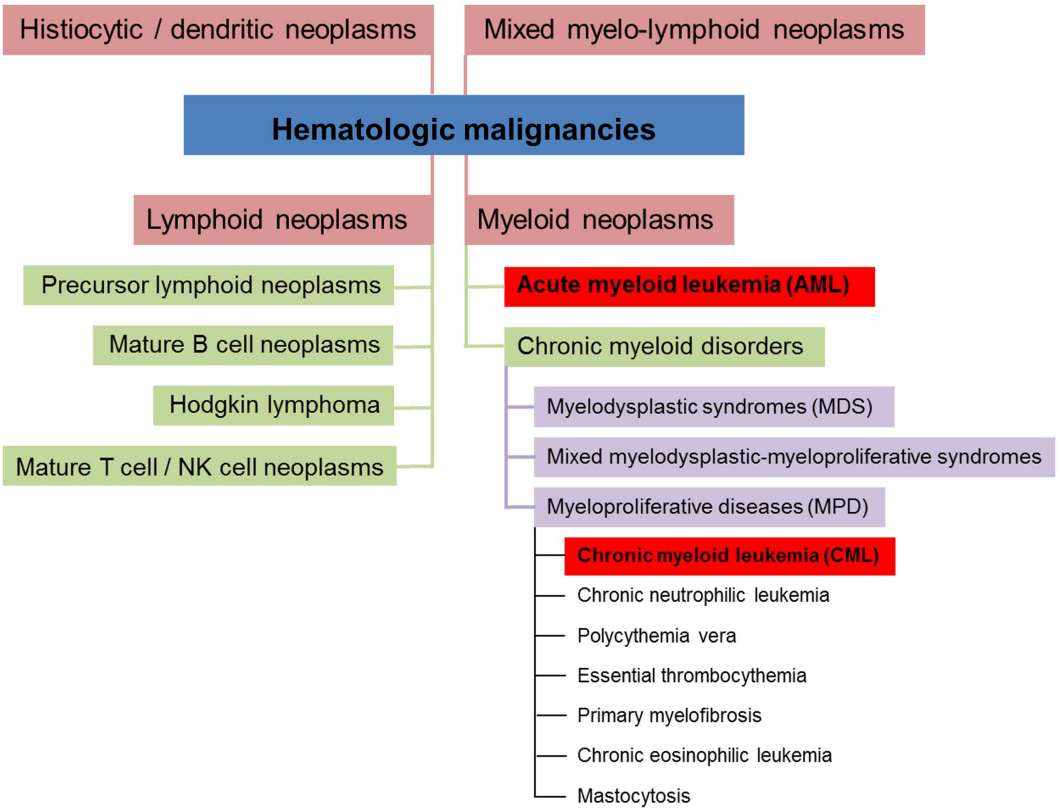

FIGURE 1 | Conceptual classification of hematologic neoplasms [based on data from Ref. (5, 6)].

in the formation of the constitutively active tyrosine kinase BCR/ABL1 and the subsequent generation of an LSC (8). CML is characterized by the overproduction and accumulation of mature, functionally impaired myeloid cells, predominantly granulocytes. CML represents about 15–20% of all leukemias in adulthood, affecting slightly more men than women (ratio ~1.8–1) (9). Its annual incidence is 1–2 cases per 100,000 for all age groups (10). This incidence is rising with age to 10–12 cases per 100,000 for people older than 80 years of age (**Figure 2**) (11). Without treatment, chronic phase CML inevitably evolves via an accelerated phase (12) into blast crisis, which is characterized by the presence of ≥20% blasts in the blood or BM or the presence of extramedullary infiltrating blasts. In two thirds of cases, the blasts are of myeloid origin and the disease phenotype is similar to AML. The other third is of lymphoid origin. Blast crisis CML is highly resistant to treatment, and median survival of patients is approximately 4–8 months. The most common causes of death in blast crisis CML are bleedings and infections due to lack of a functional hematopoietic system (13). Because BCR/ABL1 is necessary and sufficient for the malignant phenotype, attempts to inhibit this kinase using small molecules have led to the discovery of the specific tyrosine kinase inhibitor (TKI) Imatinib (14). Since its introduction into clinics in 2001, Imatinib became the gold standard in CML therapy and has replaced cytarabin/interferon (IFN)-α combination therapy (15). Imatinib is the first-line therapy of choice for nearly all newly diagnosed CML patients (16). Second- and third-generation TKIs

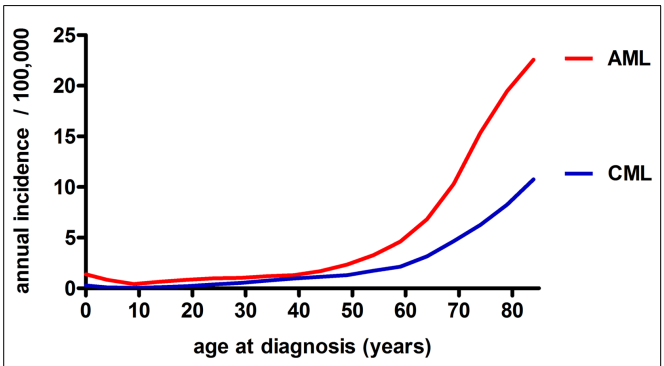

FIGURE 2 | Annual incidence of AML and CML in the USA among different age groups (both sexes, all race groups, years 1992–2010), based on data from the NCI/SEER (11).

with superior efficacy, also against mutated forms of BCR/ABL1, are currently tested in clinical trials (17–20). Even though TKI treatment stabilizes the disease during the chronic phase, a small percentage of patients will progress to accelerated phase and blast crisis (21). Besides TKIs, which have demonstrated long-term disease control and very good tolerability, the only other treatment option that may be considered for CML is aHSCT. Today, aHSCT is the only treatment with proven ability to cure CML (22).

In contrast to CML, AML is an aggressive and fatal disease caused by an increased proliferation and a block in differentiation capacity of myeloid blasts. With an annual incidence of three to five cases per 100,000 (m:f ratio, 3:2), AML is the most frequent myeloid leukemia in adults (10). Compared to CML, the age-dependent rise in AML incidence is even more drastic to peak at 20–23 cases per 100,000 in the geriatric population (**Figure 2**) (11). Besides age and sex, known risk factors for myeloid leukemias include exposure to ionizing radiation, benzene (e.g., cigarette smoking) and previous cytotoxic chemotherapy (23, 24). Despite the tremendous efforts that have been made to classify AML based on cytogenetic and molecular markers (25), AML treatment basically remained unimproved in the last 20 years and consists of induction cytotoxic chemotherapy ("3 + 7" scheme with cytarabin and an anthracycline), with minor modifications for elderly patients, therapy-related AML, and relapsed or therapy-resistant disease. The only exception is *t*(15;17)-associated acute promyelocytic leukemia (APL), which is treated with a differentiating agent, all-trans retinoic acid (ATRA) in combination with standard chemotherapy (23, 26). In face of these highly toxic chemotherapies, on average less than 30% of AML patients survive long-term. The prognosis for "elderly" patients (defined by the age of 65 or more in most studies) is even more dismal. Treatment failure may occur due to therapy-related complications, such as infections, toxicity, and tumor lysis syndrome. More importantly, the high disease relapse rate after a first remission is the major problem clinicians are confronted with in AML therapy (23). Relapse is thought to be caused by a therapy-resistant neoplastic cell reservoir slumbering in the BM, a situation referred to as minimal residual disease (MRD). It is likely that MRD represents the persistence of quiescent, therapy-resistant LSCs in the BM. Therefore, after a first remission is achieved, post-remission chemotherapy and/or aHSCT is needed to control LSCs (27).

LEUKEMIA STEM CELLS AND THE PROBLEM OF MINIMAL RESIDUAL DISEASE

The goal of therapy in myeloid leukemia is to induce a durable complete remission (CR). For chronic phase CML, this is most often relatively easily achieved by TKI treatment; however, this therapy only eliminates the bulk of leukemia cells, whereas LSCs are spared. It is thought that CML LSCs are not completely addicted to BCR/ABL1, and several studies have shown survival of CML LSCs in the presence of Imatinib *in vitro* and *in vivo* [(28) and reviewed in Ref. (20)].

For AML, induction poly-chemotherapy may result in a labile CR that has to be consolidated by aHSCT or post-remission chemotherapy. If this treatment is omitted, relapse will often occur rapidly due to persistence of MRD below the cytological detection limit of ~10^9 cells (23).

Whereas CML LSCs are relatively well characterized as lineage-negative (lin^-) $CD34^+CD38^-$ cells, the definition of the immunophenotype of AML LSCs is currently controversially discussed. Generally, LSCs are defined as a rare cell population with the capability of self-renewal, extensive proliferation, induction of leukemia, and serial transplantation capacity in xenografts as well as resistance to various treatments. Seminal studies by John Dick et al. using severe combined immunodeficiency (SCID) or non-obese diabetic (NOD)/SCID mice in the 1990s revealed that AML stem cells reside within the lin^- $CD34^+$ $CD38^-$ fraction, as the initiation of AML of all subtypes (except APL) was only possible with purified lin^- $CD34^+$ $CD38^-$ cells, but not with purified lin^- $CD34^+$ $CD38^+$ cells. The leukemias produced in these mouse models closely resembled the original human diseases, providing evidence that AML stem cells have long-term self-renewal capability and determine the leukemia's phenotype (29, 30). Based on these experiments, the authors hypothesized that leukemias are hierarchically organized in a similar way as the normal blood-forming system and that the normal HSC would most likely be the cell-of-origin that is malignantly transformed during leukemogenesis. Subsequently, many groups tried to refine the immunophenotype of AML LSCs, and several additional markers were characterized (31–36). However, findings from a recent study by Sarry et al. have questioned this strict definition of LSCs by immunophenotype. These authors showed that CD34 expression in AML is highly variable, classifying their patients into 3 groups based on the extent of CD34 expression. Importantly, LSCs were found in all samples, even in CD34 negative ones, and in some patients also in a cell population expressing low amounts of lineage markers. Therefore, these authors suggest that the absolute distribution of LSCs does not necessarily correlate with their phenotypic distribution so that even though LSCs are enriched in certain fractions of cells, such as $lin^{neg}CD38^{neg}$ cells, the relative rarity of these populations implies that the absolute number of LSCs may be higher in other cell fractions (37). In addition, the incubation of leukemia cells with antibodies targeting surface markers, such as anti-CD38, may reduce the engraftment capacity of leukemia-initiating cells expressing these markers, even further complicating the analysis of human LSCs (37, 38).

In addition to the challenging task of characterizing an LSC phenotype in AML, there is no standard definition for MRD. MRD may well serve as an indicator for the quality of the response to the treatment and may be a prognostic parameter for disease relapse and the choice and effectiveness of post-remission treatment strategy (39). Whereas CR is conventionally defined by pathologists as the absence ($\leq$5%) of blasts in the BM, the establishment of a definition for MRD is much more difficult. First, a significant proportion of AML patients lack molecular markers, such as FLT3-ITD, NPMmut, or chromosomal translocations that would allow monitoring MRD by molecular methods after induction chemotherapy. Second, the time point at which patients should first be tested for MRD and the time interval of serial monitoring is controversially discussed (40). Feller et al. suggested an interval of 3 months for MRD testing by flow cytometry (41). Third, the best method to quantify MRD is still a matter of debate. At the moment, real-time RT-PCR for molecular markers and immunophenotype using multi-parameter flow cytometry are comparable in terms of sensitivity and specificity; however, therapy-related changes in these parameters may limit the clinical applicability (42). Fourth, the level of transcript as measured by RT-PCR or number of cells as measured by flow cytometry defining the threshold for MRD^+ vs. MRD^- has to be validated in prospective studies. And last, the question remains whether peripheral blood can replace BM as the source of cells, which is

a relative prerequisite for the feasibility of such studies (39, 40). In summary, all these questions should be addressed during the design of future studies on MRD therapy.

MYELOID LEUKEMIAS AND THE IMMUNE SYSTEM

Clinical and experimental observations suggested that myeloid leukemias are partly controlled by the immune system (43). Leukemia cells express major histocompatibility class (MHC)-I and -II molecules and co-stimulatory ligands, such as CD80 and CD86, and therefore may be recognized by T cells and induce potent T cell responses (44–48). In addition, myeloid leukemias respond to unspecific immune-mediated therapies such as IFN-α and interleukin (IL)-2 (49, 50). Furthermore, aHSCT, a treatment with proven ability to cure myeloid leukemias, is in fact an immunotherapy exploiting the allogeneic T and NK cell-mediated GvL effect, which is absent in syngeneic HSCT (22, 51, 52). In addition, it was shown that patients receiving T cell depleted aHSCT grafts had a greater risk of disease relapse, and DLIs from original donors led to CR in most of the patients suffering from disease relapse (53–56).

An interesting example of endogenous immunosurveillance was observed in non-transplanted pediatric AML patients. Montagna et al. demonstrated that stable remission after cytotoxic chemotherapy was associated with the emergence of leukemia-specific CTL precursors in the BM. All patients that had high numbers of CTL precursors remained in remission, whereas the majority of patients with no CTL precursors relapsed (57).

Leukemic cells can be controlled either via specific major histocompatibility complex (MHC-restricted) mechanisms or via less specific incompatibilities in minor histocompatibility genes (58). Indeed, CTLs directed against leukemia antigens have been detected in the peripheral blood of chronic phase CML patients (59, 60) and have been shown to kill CML target cells *in vitro* via Fas-receptor triggering (61). Similar anti-leukemic CTL responses have also been documented in AML (62). In contrast, blast crisis CML cells are refractory to Fas-ligand induced apoptosis, regardless of the expression levels of Fas-receptor, suggesting that an immune-mediated selection by CTLs could result in the acquisition of Fas resistance (63). A further line of evidence that CML is controlled by CTLs comes from our own studies in a murine CML model using the glycoprotein of lymphocytic choriomeningitis virus (LCMV) as a model tumor-antigen. CML-specific CTLs were present in CML-bearing mice and displayed an exhausted phenotype as analyzed by low cytotoxicity, absence of IFN-γ and TNF-α production and expression of programed death-1 (PD-1). Nevertheless, these CTLs contributed to disease control, as depletion of $CD8^+$ T cells led to rapid disease progression and death (64). We documented that leukemia-specific CTLs are able to interact with and kill CML LSCs *in vitro* and *in vivo* in a setting with minimal leukemia load. In contrast, in a clinically relevant setting of high leukemia load, CTLs did not kill LSCs but promoted their proliferation by secreting high amounts of IFN-γ (65). In addition, we demonstrated that CD70-expressing T cells stimulate CD27-expressing LSCs in a cell-contact-dependent manner: ligation of CD27 on LSCs by CD70 on T cells reinforced the Wnt-pathway in LSCs, leading to LSC proliferation and disease progression (66). Thus, as it has been shown for other tumors, the immune system interacts with leukemia (stem) cells and may as well play a paradoxical role in promoting disease progression (67).

The role of $CD4^+$ T cells in the control of CML has been studied less intensively (68). $CD4^+$ T cells isolated from the BM of CML patients were able to suppress autologous hematopoietic progenitor cells in a contact-dependent manner (69). DLIs, depleted of $CD8^+$ T cells to reduce the side effects of GvHD, were able to induce remissions in aHSCT-treated CML patients after disease relapse. This led to the hypothesis that $CD4^+$ T cells are the main effectors of the GVL effect, whereas $CD8^+$ T cells are mainly responsible for GVHD (70). Endogenous $CD4^+$ T cells, however, might be dysfunctional *in vivo*, as they have a normal intrinsic cytokine-producing ability only *in vitro*, but not in the leukemia environment (71). However, $CD4^+$ T cells may be important in the setting of aHSCT. $CD8^+$ T cell-depleted DLI, administered to patients after aHSCT, induced a low rate of remissions and of GvHD (70). Therefore, $CD4^+$ T cells are also potentially involved in the GvL effect in CML patients. On the other hand, $CD8^+$ T cells may serve as important effectors of GvHD without being essential for GvL.

The roles of B cells and NK cells in the control of CML remain controversial. BCR/ABL1 junctional peptides could induce production of specific antibodies to BCR/ABL1 (72). In addition, it was noted that $CD4^+$ DLIs increased the numbers of circulating B cells in patients at the time of clinical response (73). Although antibodies recognizing many distinct leukemia antigens were discovered (74), the impact of antibodies on malignant CML cells remains elusive. NK cells were shown to selectively lyse CML progenitor cells *in vitro* (75). In accelerated CML and blast crisis, NK cell frequency, proliferation, and lytic function seems to decline, but it is currently unclear whether this decline is a cause rather than an effect of disease progression (76, 77). Moreover, donor-vs.-recipient NK cell alloreactivity could eliminate leukemia in human transplants (78).

Chronic myeloid leukemia patients have significantly reduced numbers of circulating myeloid and plasmacytoid DCs (pDCs) compared to healthy volunteers (79, 80). However, BCR/ABL1-expressing DCs have been detected in the peripheral blood of CML patients suggesting that CML derived DCs may possibly contribute to anti-leukemic immunity (81, 82). BCR/ABL1-expressing DCs could be generated from peripheral blood mononuclear cells (PBMCs) or $CD34^+$ progenitor cells of CML patients and were shown to have an impaired capacity to capture and process antigens and an impaired migratory capacity compared to DCs derived from healthy controls (83–85). In addition, leukemic DCs were shown to produce TNF-α and IL-8 (86). However, contradictory results about the maturation status of BCR/ABL1 DCs have been published (81, 82).

In summary, it seems plausible that innate as well as adaptive immunity play an important role in the control of myeloid leukemias.

IMMUNE EVASION MECHANISMS

Myeloid leukemias employ several strategies to compromise anti-leukemic immune responses. DCs originating from myeloid leukemia progenitor cells have been found *in vivo* in leukemia patients and were shown to be abnormal in numbers and function

(80–82, 87). Leukemia-derived DCs (L-DCs) displayed reduced antigen-capture and processing capacity, a low maturation status and an aberrant homing pattern when compared to normal DCs (86, 88). Furthermore, L-DCs promoted T cell anergy and the generation of regulatory T (T_{reg}) cells instead of inducing CTLs (89–91). T_{regs} are increased in myeloid leukemias (92, 93), are associated with an unfavorable outcome (94), correlate with disease relapse after aHSCT (95) and impede the function of adoptively transferred CTLs (96). Leukemic blasts express high levels of co-inhibitory molecules and interact poorly with T cells due to an impaired formation of immune synapse (97, 98). AML and CML cells for example express the ligands for programed death-1 (PD-L1, PD-L2), which interact with PD-1 expressed on T cells (64, 65, 99, 100). Accordingly, we recently demonstrated that CML LSCs express PD-L1 and PD-L2 as well (65). A further mechanism leukemic cells use to interfere with the immune system is the presentation of MHC class II-associated invariant chain-derived peptide (CLIP). CLIP expression on AML blasts predicts poor clinical outcome (101) and disturbs the activation of leukemia-specific $CD4^+$ T cells (102), most probably by interfering with the loading and presentation of LAAs (103). Interestingly, CLIP could also promiscuously bind to various MHC class I types in leukemia cells deficient of MHC class II, a feature that could hamper CTL-mediated leukemia immunosurveillance (104).

The role of tumor necrosis factor (TNF) and TNF-receptor superfamily members in the pathophysiology of leukemia has recently been documented. Glucocorticoid-induced TNFR-related protein ligand (GITRL) was shown to be expressed in a majority of AML cell lines and blasts from patient samples. Reverse signaling of GITRL in AML cells induced the release of TNF and IL-10, and triggering of GITR expressed on NK cells impaired NK cell cytotoxic function and IFN-γ production (105). AML cells exploit further signaling axes of the TNF/TNFR superfamily, such as the 4-1BB-ligand/4-1BB (CD137L-CD137) pathway and the receptor activator of nuclear factor kappa B (RANK)-ligand RANK pathway (106, 107) to inhibit the immune system in a similar way as described for GITR. Furthermore, we could recently document a role for the TNFR CD27 on proliferation of CML LSCs and CML progression (66). Blocking inhibitory pathways holds promise for clinical development. Among them are FAS-ligand that induces apoptosis of FAS-expressing T cells, CD200 that directly inhibits T and NK cells, reactive oxygen species (ROS) that induce lymphocyte apoptosis, killer-cell immunoglobulin-like receptors (KIR) that suppress NK cells and indoleamine 2,3-dioxygenase (IDO) that depletes tryptophan required for T cell expansion or IL-10 that potently suppresses T cell activation [reviewed in Ref. (27)]. Besides inhibiting the adaptive immune system, it was recently demonstrated that leukemic cells are able to block programed cell removal by innate immune cells, thereby overcoming a further regulatory mechanism that normally limits cancer growth. The up-regulation of so-called "don't eat me" signals on blasts and leukemia stem cells (LSC), such as CD47 and CD200, precludes apoptosis-independent phagocytosis by macrophages. In addition to enable the propagation of the malignant cells, this mechanism likely allows metastatic circulating cancer cells to survive in niches rich in phagocytes, such as the spleen and lymph nodes (108, 109).

These and further immunosuppressive mechanisms remain major hurdles to be overcome in order to successfully implement DC-based immunotherapy in the treatment of leukemia. Interfering with negative immune regulators may effectively improve DC-based immunotherapy, as has been shown by silencing the suppressor of cytokine signaling 1 (SOCS1) or the immunosuppressive cytokine IL-10, which enhanced antigen-presentation and secretion of IL-12 by DCs and triggered an effective anti-tumoral immune response (110–112).

CROSS-PRIMING OF CTLs BY DCs

Cross-presentation is fundamental for the maintenance of peripheral tolerance and the induction of cross-priming. The concept of cross-presentation defines the processes of antigen uptake and processing and presentation on MHC class I by professional APCs to CTLs (113, 114), whereas cross-priming describes the stimulation and activation of naïve CTLs by this process (115). According to our current understanding that is primarily derived from viral infection models, CTL cross-priming takes place in secondary lymphoid organs (116). Antigen-experienced, matured DCs migrate and transport the viral antigen from the infection site for cross-presentation to secondary lymphoid structures (117).

The crucial factor for DCs to tune CTL activation is their maturation status (118). Several studies demonstrated that the presence of appropriate inflammatory and co-stimulatory maturation signals, such as pathogen-associated molecular patterns (PAMPs), TLR ligands, type I IFNs, CD80/CD86, and CD70 (119, 120) as well as CD40 ligand (CD154) provided by $CD4^+$ T cells ("DC licensing") is essential for DCs to properly activate CTLs in viral infections (118). It is well documented that solid and hematological tumor microenvironments contain DCs in mice and men [reviewed in Ref. (121)]. These microenvironments, however, lack DC-activating and DC migration-inducing factors (122) and harbors a multitude of immunosuppressive molecules such as TGFβ and IL-10 that impair DC maturation, migration and antigen (cross-) presentation [reviewed in Ref. (123)]. AML blasts can generate an immunosuppressive microenvironment that hinders effective adaptive as well as innate immune responses (124–127), such as by the secretion of arginase II resulting in T cell inhibition (124). Cross-presentation of the LAAs proteinase-3 and PR1 has also been shown in AML patients, but these antigens were presented by immature DCs resulting in tolerization of CTLs (128).

Therefore, even though there is compelling evidence that LAAs are cross-presented *in vitro* and *in vivo*, the question as to what extent the process of cross-priming contributes to anti-leukemic immunity is still highly controversial (114).

Nevertheless, fully functional CTLs are fundamental for the surveillance, control, and elimination of tumors (129, 130). Therefore, a better understanding of specific DC subsets in the anti-leukemic immune response and how cross-presentation of LAAs *in vivo* can be improved and consequently CTL dysfunction circumvented, may lead to improved vaccine-based immunotherapy against leukemia.

LEUKEMIA ANTIGENS

In order to achieve an optimal and effective immune response with a low rate of toxicity, leukemia antigens that are specifically expressed and presented by leukemia cells and not by healthy tissue have to be identified. In addition, these should be immunogenic

and should critically account for the leukemic phenotype. Most importantly, however, these antigens should be expressed in LSCs, even though currently the phenotypic characterization of LSCs is controversial and elusive. The restricted numbers of clearly identified LAAs in leukemia remain a major obstacle for the use of these peptides in DC-based immunotherapy. In addition, the low affinity of these LAAs to bind MHC I, the short time of antigen-presentation on DCs as well as the lack of help by $CD4^+$ T cells may limit the capacity of these LAAs to induce an anti-tumoral immune response (131, 132).

The most specific leukemia antigens are peptides from aberrant proteins created by mutations or translocations only present in leukemia cells, such as the BCR/ABL1 tyrosine kinase in CML. These peptides are known as leukemia-specific antigens (LSAs). However, most of the leukemia-specific mutations and translocations do not give rise to proteins (133). Among the numerous chromosomal translocation that were characterized in AML, only a minor fraction such as AML1-ETO (133), DEK-CAN (134), and PML-RARα (135) gives rise to proteins that generate LSAs. In addition, only two mutations involving the fms-related tyrosine kinase (FLT) and nucleophosmin 1 (NPM1) have been shown to give rise to LSAs (136, 137). Therefore, most immunotherapy approaches in myeloid leukemia target LAAs, that is, peptides from proteins that are expressed in leukemic cells and also healthy tissues, but are often overexpressed in leukemia and important for the malignant phenotype. Consequently, the induction of autoimmunity is a potential risk if such LAAs are chosen as targets for an immunotherapy. As an additional limitation, T cell receptors recognizing antigens that are broadly expressed on healthy tissues in the body are usually of low affinity. Therefore, it is crucial to characterize the degree of LAA expression on normal tissues in order to envisage the multitude and characteristics of potential autoimmune reactions.

For AML, a multitude of LAAs has been described during the last two decades and has been validated as target for immunotherapy [**Table 1** and reviewed in Ref. (133)]. These LAAs comprise proteinase-3, Wilms tumor protein (WT1) (62, 138–141), the receptor for hyaluronic acid-mediated motility [RHAMM/CD168 (142)] human telomerase reverse transcriptase [hTert (143)], preferentially expressed antigen in melanoma [PRAME (144, 145)], and Aurora-A kinase (146) (**Table 1**).

The most attractive and promising LAA is the tumor-suppressor gene and zinc finger transcription factor WT1. WT1 is a regulator of cell proliferation, differentiation, and apoptosis. In leukemia, WT1 has been shown to have a fundamental oncogenic role for leukemogenesis resulting in differentiation arrest and aberrant cell growth (147). WT1 was demonstrated to be immunogenic as it elicits a naturally occurring anti-tumoral immune response in cancer patients (148, 149). In addition, in a WT1 directed immunotherapeutic study, off-target toxicity effects have not been observed, indicating that WT1-expressing normal tissues are omitted from the response (150). However, in some AML patients no WT1-specific CTL response has been triggered even though objective responses and remissions have been elicited (141). Importantly, WT1 is expressed to a much lesser extent on normal HSCs than on leukemic blasts and LSCs in a majority of AML patients which characterizes WT1 as attractive target for immunotherapy in AML (151, 152). Consequently, WT1 is currently targeted in clinical T cell therapy and vaccination studies.

Table 1 | Leukemia-associated antigens (LAAs) in myeloid leukemias.

Myeloid leukemia	LAA	Reference
AML	Aurora-A kinase	(146, 153, 154)
	BRAP	(160)
	Cyclin A1	(161)
	hTert	(143)
	HSJ2	(160)
	MPP11	(160)
	Neutrophil elastase (NE)	(166)
	PRAME	(144, 145, 162)
	PR1	(128, 139, 163, 164)
	Proteinase-3	(62, 164, 165)
	RBPJκ	(160)
	RHAMM/CD168	(142)
	WT1	(62, 139, 141, 148, 149, 151, 152)
CML	BRAP	(160)
	CML-28	(167–169)
	CML-66	(167–169)
	HAGE	(168)
	HSJ2	(160)
	MPP11	(160)
	PRAME	(144)
	PR1	(59, 139, 164, 169)
	Proteinase-3	(164, 165, 169)
	RBPJκ	(160)
	Survivin	(167–169)
	WT1	(139, 148, 149, 169–171)

AML, acute myeloid leukemia; BRAP, BRCA1-associated protein; CML, chronic myeloid leukemia; HAGE, helicase antigen; HSJ2, heat-shock 40 kDa protein 4; hTert, human telomerase reverse transcriptase; MPP11, M-phase phosphoprotein 11; PRAME, preferentially expressed antigen in melanoma; RBPJκ, recombination signal binding protein for immunoglobulin kappa J region; RHAMM, receptor for hyaluronic acid-mediated motility; WT1, Wilms tumor protein.

Importantly, in a curative approach LAAs have to be expressed on LSCs (146, 153, 154). One LAA in AML that is expressed on $CD34^+CD38^-$ AML "stem" cells compared to $CD34^+CD38^+$ AML progenitor cells and normal $CD34^+$ stem/progenitor cells from healthy individuals is the serine/threonine kinase Aurora-A. Importantly, $CD34^+$ leukemia progenitor cells but not normal $CD34^+$ stem/progenitor cells were lysed by Aurora-A kinase-specific CTLs. Furthermore, blockade of Aurora-A kinase by a small-molecule inhibitors or shRNA impaired engraftment and improved survival of mice in an AML xenograft model (146, 153, 154).

In CML patients numerous LAAs such as WT1, proteinase-3, cancer-testis antigens like HAGE, minor histocompatibility antigens, hTert, CML-66, CML-28, and survivin were shown to be aberrantly expressed in the transformed CML cell (**Table 1**). Some LAAs such as hTert and survivin have a quite restricted

expression pattern and are not or only marginally expressed on normal non-dividing or terminally differentiated cells (143, 155). This makes hTert and survivin promising targets for DC-based immunotherapy.

The most prominent LSA in CML is the chimeric BCR/ABL1 fusion protein, an ideal target for immunotherapy (8). An elegant paper by Yotnda et al. identified a BCR/ABL1 junctional nonapeptide (SSKALQRPV) that binds to human leukocyte antigen (HLA)-A2.1 and elicits specific CTL responses *in vitro* in blood from healthy donors and CML patients. In 5 out of 21 CML patients, the investigators found high frequencies of junctional peptide-specific CTLs in the peripheral blood, suggesting an *in vivo*-immunogenicity of this peptide (156). Additional studies confirmed and extended the finding of immunogenic BCR/ABL1 junction peptides (157, 158). However, BCR/ABL1 is gives rise to a limited number of immunogenic epitopes due to only two chromosomal breakpoints (159). Furthermore the expression of the epitopes is restricted to HLA A2, A3, and B7 (158).

Since all the LAAs listed in **Table 1** are expressed to a greater extent on malignant cells than on their healthy counterparts, they represent suitable antigens for immunotherapeutic approaches.

IMMUNOTHERAPY FOR MYELOID LEUKEMIAS

Nowadays, immunotherapy covers a huge spectrum of distinct experimental procedures in order to specifically eliminate cancer cells while minimizing harm to normal tissue to limit side effects (172). However, up to now only few approaches have entered clinical routine such as unspecific immune stimulation by Bacillus Calmette–Guérin (BCG) instillations to treat non-muscle invasive bladder cancer after surgical ablation (173) or the immunomodulating anti-CTLA4 mAb Ipilimumab for metastatic melanoma or prostate cancer (174), as well as aHSCT for the treatment of myeloid leukemias (175) and the prostate antigen-specific DC-based vaccine Sipuleucel-T (Provenge®) for hormone-refractory prostate cancer (176).

The intention of active cancer immunotherapy is to mount an endogenous adaptive immune response against a tumor by directly injecting tumor-antigens together with adjuvants ("peptide vaccines") or by *ex vivo*-generation of cancer-specific DCs ("DC vaccines") and to form CTL memory in order to sustain remission (177). For AML and CML, numerous studies extensively investigated the clinical potential of this approach. Administration of autologous DCs loaded via electroporation with mRNA of the LAA WT1 resulted in CR in 50% of AML patients in a phase I/II study (141). Importantly, CR was achieved in two patients that only had partial remission after chemotherapy, indicating the feasibility and clinical potential of this approach. In contrast, in a clinical phase I study, autologous monocyte-derived DCs (mDCs) previously cultured in the presence of AML failed to induce a clinical response in relapsed AML patients (178).

Recently, a better understanding of immunosurveillance paved the way for the development of new immunotherapeutic approaches and their implementation in the clinics. Among these, immune checkpoint inhibition is most advanced in melanoma patients and anti-CTLA4 blockade was actually the first therapy that improved survival of patients suffering from metastatic melanoma (174). A recent hallmark immunotherapeutic study using a dual mAb treatment approach to block the immune checkpoint regulators CTLA-4 and programed death-1 (PD-1) using Ipilimumab and Nivolumab, respectively, resulted in persistent tumor regression in advanced melanoma patients (179). AML and CML cells also express the ligands for PD-1 (PD-L1, PD-L2), which interact with PD-1 expressed on T cells (64, 65, 99, 100). Accordingly, we recently demonstrated that CML LSCs express PD-L1 and PD-L2 as well (65). In addition, we recently demonstrated that blocking PD-1 signaling results in improved CML control in pre-clinical mouse models (64).

Furthermore, chimeric antigen receptor (CAR) T cells for adoptive T cell therapy (ACT) proved their clinical potential in leukemia patients. In addition, ACT with CAR T cells overcame the obstacle to generate sufficient numbers of high avidity LAA-specific T cells *in vitro* and long-term persistence, memory formation, and migration *in vivo*. Chronic lymphocytic leukemia (CLL) patients treated with a low number of CAR T cells targeting CD19 and containing the co-stimulatory signaling domain of CD137 exhibited a CR. Importantly, CAR T cells extensively expanded and showed a CD19-specific immune response as well as long-term persistence with an effector memory phenotype in peripheral blood and BM without the need to trigger an anti-leukemic immune response by professional APCs. This phenotype consequently allows potential expansion upon secondary encounter with CLL cells and prevention of relapse (180). Furthermore, two children with relapsed and refractory pre-B cell ALL treated with CD19-specific CAR T cells were reported to have achieved CR (181). For myeloid leukemias a clinical application of CAR T cells has not yet been documented. However, CAR T cells targeting isoform 6 of CD44 (CD44v6) that is expressed by AML cells (182) but not by HSCs and at low levels on normal cells (183) mediated potent anti-tumor effects against primary AML in a pre-clinical AML model (184). In addition, clinical phase I/II studies (NCT01640301, NCT01621724) using ACT of T cells carrying a TCR specific for the LAA WT1 in AML patients are ongoing. These trials are essential to further determine if safety and efficacy of this promising immunotherapeutic approach also holds true for the treatment of AML patients.

DC-BASED IMMUNOTHERAPY IN LEUKEMIA

Because of their excellent ability to activate T cells, DCs are considered as one of the most promising tools for tumor-antigen delivery in active cancer immunotherapy and they are ideal candidates to supply foreign tumor-antigen in the form of a DC-based vaccine or for the generation of tumor-antigen-specific CTLs *in vitro* (185). Clinical studies have used various precursor cells in order to manufacture sufficient *ex vivo* tumor-antigen loaded DCs for immunotherapeutic purposes (186). However, the different methods in manufacturing those DCs and the notion that the generated DCs differed in function and phenotype resulted in need for the standardization of DC vaccines.

To vaccinate AML patients with DCs in order to induce an optimal, long-lasting anti-leukemic CTL response, several issues have to be considered:

First, the type and origin of DCs used to treat the patient has to be defined. DCs can either be generated from patient-derived $CD34^+$ cells or $CD14^+$ monocytes *in vitro*. They can be directly positively selected from the patient's PBMCs (*ex vivo*) and are differentiated in the presence of various cytokines which improves

the LAA loading onto these DCs (177). Additionally, naturally circulating DCs can be loaded and activated *in vivo* using mAbs targeting SIGLEC H conjugated to an LAA in combination with CpG nucleotides (187, 188). In leukemia patients, especially in AML patients, the presence of blast-derived leukemic DCs was extensively documented (80–82, 87). Consequently, a promising method of generating L-DCs is to differentiate blasts from AML patients into DCs *ex vivo*. This method allows circumventing the loading of the DCs with LAAs. The application of these AML-derived DCs in a clinical setting is still poorly developed. Especially, the generation of sufficient numbers of AML-derived DCs is challenging. Only 25% of the initial AML cells cultured can be converted into AML-derived DCs. In addition, AML-derived DCs can only be generated in around 40% of AML patients due to AML-specific mutations (e.g., Flt-ITD) or the lack of CD14 expression that prevent the conversion of blasts into AML-derived DCs (189, 190). Nonetheless, the tolerability of this therapeutic approach and the induction of an anti-leukemic immune response in patients have been already reported. Despite these positive reports, the clinical benefit of the DC vaccine is only marginal (191). Therefore, the current DC-based cancer immunotherapy protocols using AML-derived DCs are optimized and standardized in order to allow generating sufficient AML-derived DCs (192, 193) with an improved potential to prime and activate CTLs and increase their cytolytic capacity (194, 195).

The other critical factors determining the success of DCs in AML immunotherapy besides the generation of sufficient numbers of DCs are (1) the selection of the proper LAA (discussed later), (2) the method applied for loading the respective leukemia antigen onto the DCs, and (3) the strong activation of DCs necessary to provide sufficient co-stimulatory signal for efficient T cell activation and to prevent T cell tolerization.

Originally, mDCs have been cultured together with AML cell lysates or immunogenic apoptotic/necrotic AML cells to ensure LAA loading [**Figure 3** (185)]. As an additional approach, AML blast-mDC cell-fusion hybrids have been generated *in vitro* [**Figure 3** (196)]. Importantly, all these multi-epitope approaches might deliver a variety of known and unknown LAAs to the DCs. In addition, these approaches circumvent the need for previous identification of the LAAs. On the other hand, co-culturing or fusion approaches might negatively impact the antigen uptake and processing and/or the maturation of DCs because of immunosuppressive factors stored in or produced by AML cells, such as TGF-β (185). Nevertheless, Herr et al. have shown that tumor cell lysate-loaded DCs were superior to DCs loaded with eluted peptides in inducing an anti-tumoral immune response against an EBV^+ B lymphoblastic cell line (197). Nowadays, pre-clinical and clinical approaches favor the loading of DCs with peptides from LAAs or LSAs such as WT1, Survivin, PML-RARα, etc. via peptide pulsing or electroporation and mRNA loading [**Figure 3** (191)]. Most of the studies using one of these loading methods reported activation and expansion of HLA-compatible CTLs *in vitro* resulting in killing and eliminating of the leukemia cells, indicating a reasonable rationale to apply mDC immunotherapy in a clinical setting irrespective of the antigen loading method. However, the use of single antigens poses the risk of immunoediting and the escape of antigen-loss variants (198). Especially, the technique of

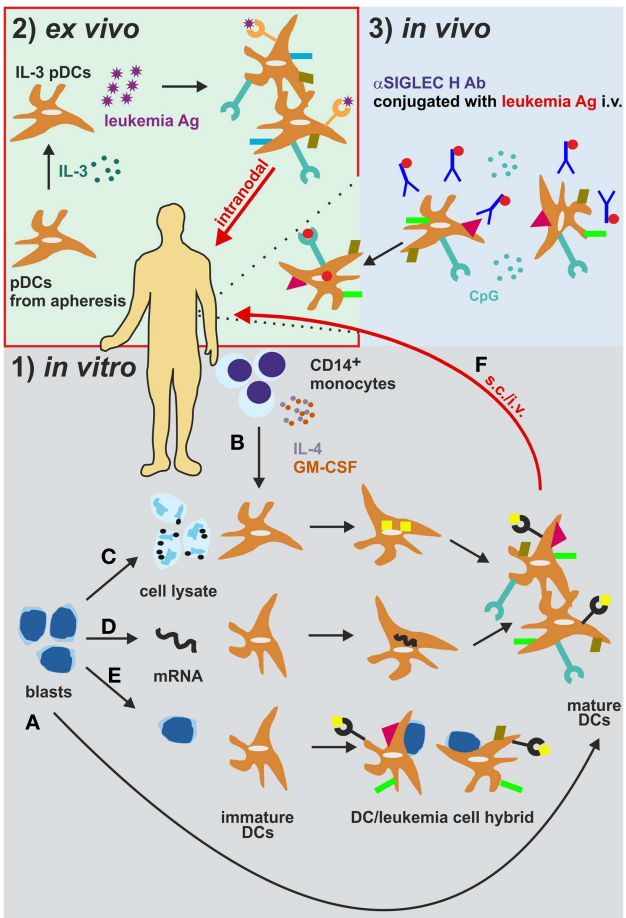

FIGURE 3 | Different strategies for the generation and administration of DC-based vaccines in AML. (1) **(A)** Leukemia-derived DCs can be directly generated by isolation and differentiation from AML blasts *in vitro*. **(B)** $CD14^+$ monocytes from patients or healthy donors are differentiated into monocyte-derived DCs (mDCs). These mDCs are cultured together with **(C)** AML cell lysates or immunogenic apoptotic/necrotic AML cells (185) or **(D)** are electroporated with mRNA from AML cells (191) to ensure leukemia antigen loading. **(E)** As an additional *in vitro* approach, AML blast-mDC cell-fusion hybrids are artificially generated (196). **(F)** The DCs are then injected s.c. or i.v. into AML patients. (2) DCs can also be loaded and activated *in vivo* (188). DCs express the endocytosis receptor SIGLEC. Intravenous administration of an αSIGLEC H mAb conjugated to a leukemia antigen in the presence of CpG results in DC activation, antigen uptake and presentation. (3) Plasmacytoid DCs isolated from AML patients are activated and loaded with leukemia antigens *ex vivo* and are re-injected intralymphatically into lymph nodes (201). Ab, antibody; Ag, antigen; i.v. intravenously; pDCs, plasmacytoid DCs. s.c., subcutaneously.

mRNA electroporation offers several advantages to overcome this issue: (1) simultaneous loading and presentation of multiple LAA epitopes without any risk for insertional mutagenesis due to the only transient mRNA expression (199); (2) expression of multiple patient-specific LAAs at once, when electroporation is performed with whole AML cell lysate mRNA (200); and (3) the possibility of combination with other loading methods (200).

For optimal DC activation and antigen processing of *in vitro* generated DCs, different cocktails of cytokines and TLR ligands

have been tested. Usually, patient-derived monocytes are cultured in the presence of IL-4 and granulocyte-monocyte colony stimulating factor (GM-CSF) for several days, followed by a short course of DC maturation using TLRs, pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, prostaglandin E2, and/or CD40 ligand (202). Similar procedures have been applied for AML blast-derived DCs (185, 203–210). However, the effects of these *in vitro* culture and maturation conditions on the ability of DCs to capture, process and present antigen, on their T cell activating potential and on their *in vivo* migratory function are not fully understood (177). For example, the replacement of IL-4 by IL-15 during the differentiation phase was shown to enhance the immunostimulatory properties of DCs with a phenotype and characteristics of Langerhans cells (LCs), which are *per se* far more efficient in antigen-presentation and T cell priming *in vitro*. (211, 212). In addition, it was demonstrated that DCs matured conventionally in the presence of pro-inflammatory cytokines are unable to produce IL-12 *in vivo*, a cytokine that is essential for $CD4^+$ T_H1 cells differentiation. Maturation in the presence of the TLR7/8 agonist R848 restored IL-12 secretion, improved cell migration and led to more robust induction of anti-leukemic immune responses *in vitro* (202, 213, 214).

Tracking of labeled and intradermally administered DCs in patients revealed that less than 1% of the DCs are migrating into the adjacent lymph nodes (215). In order to circumvent the drawbacks of *in vitro* DC generation and their poor migration into lymph nodes, in a clinical study of DC-based immunotherapy in melanoma, Tel and colleagues directly isolated human pDCs and injected them intralymphatically into the inguinal lymph nodes after *ex vivo* activation and loading (201). pDCs, specialized DCs that are characterized by rapid and massive secretion of type I IFNs in response to foreign nucleic acids, have been shown to successfully mediate an interplay of innate and adaptive immune responses by activating other DCs and inducing cross-priming (216–218). Compared to subcutaneous injections, intralymphatic immunotherapy substantially reduces the amount of vaccine necessary and the duration of immunization. This approach has already proven effective for the treatment of allergies [**Figure 3** (219)]. Therefore, pDCs and/or intralymphatic injection protocols may become crucial players in eliciting anti-leukemic immunity.

By now, it is unfortunately not fully elaborated which DC subset is most suitable for DC-based immunotherapy. The identification of this subset, the optimal route of administration, the optimal dose, the optimal antigen, and conditioning in order to maximize the efficacy of the treatment is pivotal for the success of treatment. Therefore, future studies have to fully aim at the functional characterization of different DC subsets in terms of T cell (cross-) priming, migration capacity, cytokine production, half-life etc. in order to maximize the clinical benefit of the therapy. The fundamental challenge in the treatment of AML remains the prevention of clinical relapse of the patients. The generation of clinical grade AML-derived DCs from AML patients in remission has been reported (220) and may consequently serve as a potential strategy in order to avoid a potential relapse (**Figure 4**). In addition, results from recent clinical phase I/II studies treating AML patients in remission with clinical grade DCs generated with different protocols highlight the importance of the selection of the antigen, the loading approach as well as the time of administration as fundamental success criteria for DC-based immunotherapy in AML.

CLINICAL TRIALS

Currently, several peptides derived from LAAs are under clinical investigation for myeloid leukemia patients in current vaccination trials. Ongoing or recruiting DC vaccination trials in phase I and II use either different WT1 derived peptides (NCT01686334, NCT00834002, NCT00672152, NCT01266083), the proteinase-3-derived peptide PR1 (NCT00454168), the peptides MAGE-A1, MAGE-A3, and NY-ESO-1 (NCT01483274) or a combination of WT1 and PR1 (NCT00433745, NCT00488592). These trials primarily include patients that just underwent aHSCT, elderly patients or patients in first remission. Interestingly, one study that has been completed recently applied a vaccination protocol with lethally irradiated autologous AML cells that were genetically modified to secrete human GM-CSF in order to enhance LAA presentation (NCT00136422). Another trial that aimed at up-regulating LAA presentation additionally administered the hypomethylating drug decitabine (NCT01483274). More and more studies use DC vaccination in combination with other drugs or cytokines. For example, in a clinical phase II study, CML patients in remission are treated with PR1 peptide vaccine in combination with pegylated IFN-α2b (PegIntron®, NCT00415857). Another approach combines a DC cell/AML fusion vaccine with the blockade of PD-1 (NCT01096602).

All these clinical trials have proven that DC-based immunotherapies in leukemia are safe and have hardly any side effects. Unfortunately, this good tolerability is accompanied by a rather minor clinical benefit in terms of response rate or other important clinical outcome parameters (191). From immunotherapy trials in solid tumors we have learned that the established response criteria for chemotherapy, such as the "RECIST criteria," may not be appropriate for immunotherapy approaches. This may also hold true for leukemia. Reduction of leukemia load or remission in the BM shortly after the treatment may not be the appropriate readout to judge the efficacy of an active immunotherapy that needs time to be established and may contribute to a long-term control of the disease. In addition, suitable biomarkers that are predictive for a response to an immunotherapy are still lacking (177). Therefore, future studies also have to focus on the generation of adequate readouts and the identification of defined biomarkers for the monitoring of DC-based immunotherapy in leukemia. Furthermore, most clinical studies carried out so far enrolled leukemia patients with a high leukemia burden. In these studies, at least some of the patients showed a minor clinical benefit. Importantly, applying DC-based immunotherapy to patients with a lower leukemia burden or MRD might result in better responses.

CONCLUDING REMARKS

During the last decade, the combined efforts of researchers to treat myeloid leukemia unraveled a multitude of LAAs suitable for DC-based immunotherapy. Consequently, DC-based immunotherapy slowly progresses into the clinical treatment of leukemia.

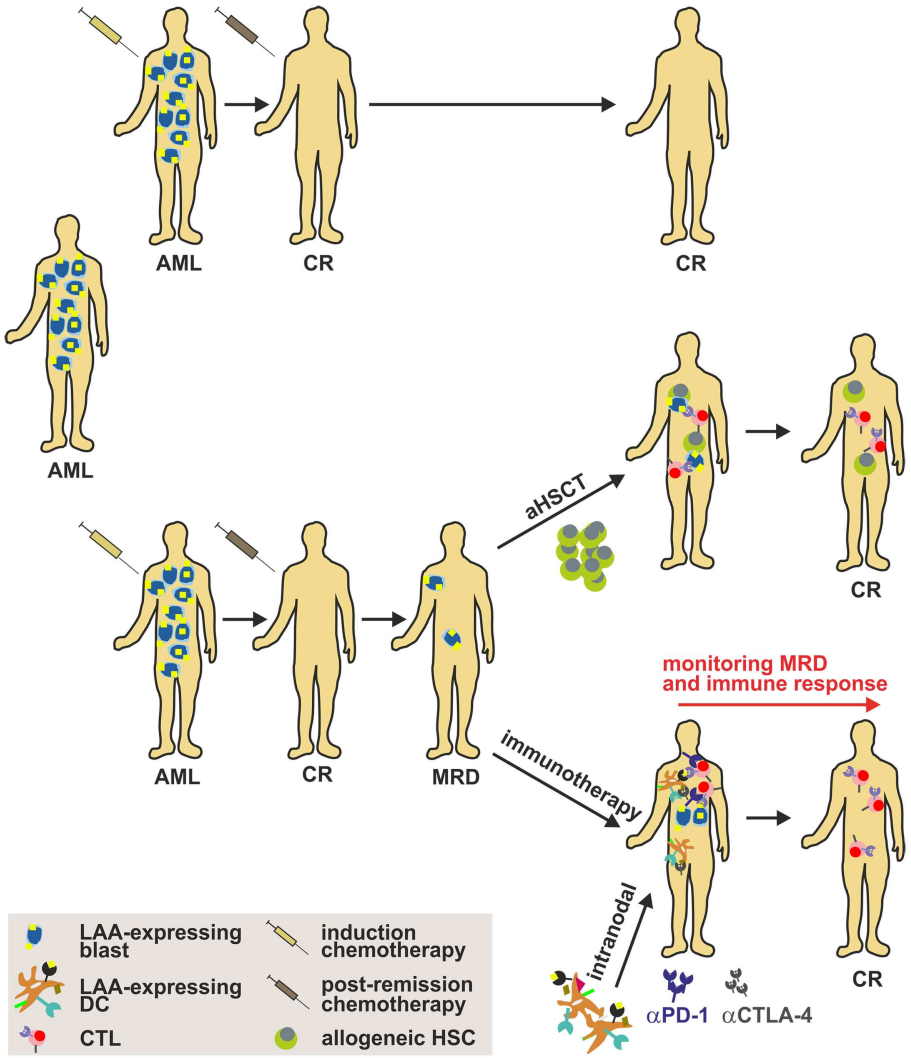

FIGURE 4 | Strategy to implement DC-based immunotherapy in the treatment of AML. Induction cytotoxic chemotherapy ("3 + 7" scheme with cytarabin and an anthracycline) results in a labile complete remission (CR) that has to be consolidated by post-remission chemotherapy. Nonetheless, many patients harbor persistent LSCs after a CR (referred to as MRD), which may cause disease relapse. Therefore, strategies such as aHSCT (only for a minor fraction of patients) or immunotherapy have to be implemented to sustain CR. Importantly, DC-based immunotherapy targeting AML-specific LAAs alone or in combination with immune checkpoint inhibitors such as anti-CTLA-4 or anti-PD-1 mAbs might be a promising approach to treat patients and to target and eliminate LSCs. aHSCT, allogeneic hematopoietic stem cell transplantation; AML, acute myeloid leukemia; CTL, cytotoxic lymphocyte; CR, complete response; DC, dendritic cell; LAA, leukemia-associated antigen; MRD, minimal residual disease.

The rapid development in the field allowed the design of phase I and II studies with different DC vaccination protocols. DC-based vaccination often resulted in the induction of potent anti-leukemic CTL responses. The benefit for the patient in these studies in terms of response to treatment was rather limited. Nevertheless, DC vaccination protocols remain a promising supplementary strategy in the treatment of leukemia, and future improvements will reveal their full potential. In order to improve DC-based vaccination for clinical routine, several issues still have to be solved. Most importantly, an optimal timing for the vaccination during the course of disease has to be defined. Current literature and our own experiments indicate that immunotherapy may be most effective in the state of MRD after successful induction and post-remission chemotherapy. In parallel, MRD has to be better defined, characterized, and especially quantified by the introduction of more sophisticated molecular and flow cytometry techniques. Simultaneously, it is of extreme importance to quantitatively and functionally assess the degree of the anti-leukemic CTL response. Furthermore, the vaccination procedure, including the choice of LAA (or multiple LAAs); the source of DCs (mDCs, LCs, pDCs, or AML-derived DCs); the DC maturation protocol and the way of application (i.v. vs. s.c. vs. intralymphatical) have to be defined and standardized. Finally, the timing and application of potential co-treatments, including chemotherapy, aHSCT and immunomodulating agents has to be considered. Especially, combining DC-based immunotherapy with the blockade of immune

checkpoint regulators such as PD-1 and/or CTLA-4 may represent a powerful tool for the treatment of leukemia.

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AUTHOR CONTRIBUTIONS

Christian M. Schürch and Carsten Riether wrote the manuscript and Adrian F. Ochsenbein revised the manuscript.

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Dendritic cell vaccination, immune regulation, and clinical outcomes in ovarian cancer

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Clinical optimism for dendritic cell vaccination against ovarian cancer has been tempered by the knowledge that tumors avail themselves of multiple mechanisms of immune evasion, thus blunting the efficacy of therapeutic vaccination. Mechanisms of immune suppression include infiltration by regulatory T cells (Treg) and myeloid suppressor cell populations, expression of co-inhibitory receptors, and expression of indoleamine 2,3-dioxygenase (IDO). Expression of both B7-H1 and IDO are associated with differentiation and recruitment of Treg, and clinical studies have shown that each of these mechanisms correlates independently with increased morbidity and mortality in ovarian cancer patients. In sharp contrast, recent studies have indicated that Th17 cell infiltration in ovarian cancer correlates with improved patient outcomes and prolonged overall survival. Given that IDO plays a pivotal role in the balance between Treg and Th17 immunity, elucidation of the mechanisms that regulate IDO activity and immune suppression may lead to novel adjuvants to boost the clinical efficacy of dendritic cell vaccination against ovarian cancer and other malignancies.

Keywords: ovarian cancer, regulatory T cells, Th17 T cells, dendritic cells, indoleamine 2,3-dioxygenase

THE CLINICAL PROBLEM

Clinical studies have shown that the immune system plays an active and possible critical role in the pathogenesis of ovarian cancer, disease progression, and overall survival. Of the positive parameters, CD3 T cell infiltration has been associated with prolonged survival (1). A notable point from this investigation was that patients with significant T cell infiltration in their tumors were more likely to be optimally debulked during surgery provided an indication that T cells may limit regional spread of the disease. In contrast with these positive findings, the majority of studies have highlighted multiple mechanisms of immune suppression that correlate with poor patient outcomes in ovarian cancer.

Regulatory T cells (Treg) infiltration has been widely noted as a negative correlate of clinical outcomes for many malignancies, and ovarian cancer is no exception. Curiel and colleagues showed that Treg infiltration in ovarian cancer correlates with a poor prognosis and increased mortality (2). Other investigators have shown that high expression of Foxp3 (a transcription factor associated with a Treg phenotype) is an independent prognostic indicator for reduced survival (3), and that a high CD8/Treg ratio is associated with more favorable outcomes (4). Further mechanisms that contribute to the immunosuppressed state include expression of PD-L1 (B7-H1), which can promote T cell anergy and apoptosis through engagement of PD-1 expressed by effector T cells (5, 6) and expression of indoleamine 2,3-dioxygenase (IDO). Expression of both B7-H1 and IDO are associated with differentiation and recruitment of Treg (7–9), and clinical studies have shown that each of these mechanisms correlates independently with increased morbidity and mortality in ovarian cancer patients (10–12). Immune suppression in the tumor micro-environment is also likely to present a formidable barrier to the clinical efficacy of therapeutic tumor vaccination, including dendritic cell (DC) vaccination.

In sharp contrast with the evidence that Treg infiltration is associated with poor outcomes in ovarian cancer, Th17 T cell infiltration correlates with more favorable clinical outcomes (13). Furthermore, tumor-infiltrating Th17 cells were negatively associated with Treg infiltration, suggesting a reciprocal relationship between these subsets. These observations have led to the question of whether therapeutic benefit would accrue from induction or expansion of Th17 cells, either through DC vaccination, other types of tumor vaccines or adoptive immunotherapy (14, 15).

CAN DENDRITIC CELLS STIMULATE TH17 RESPONSES AGAINST OVARIAN CANCER?

The tumor micro-environment can modify DC function through multiple mechanisms, usually resulting in inhibition of DC activation and maturation, and the induction of immunosuppressive DC and related myeloid cell populations (16). Tumor inhibition of DC function can also have an impact on therapeutic DC vaccines, indicating the need for DC vaccines with the capability to redirect T cell immunity from immune suppression to pro-inflammatory anti-tumor responses. Several lines of evidence have pointed to a crucial role for MAPK signaling pathways in regulation of pro-inflammatory versus tolerogenic or immunosuppressive DC function. Notably, Jackson and colleagues demonstrated that blockade

of MEK 1/2 and ERK MAPK signaling restores tumor-mediated inhibition of DC function and promotes IL-12 production and Th1 T cell responses, whereas inhibition of p38 MAPK increases signal transduction through ERK 1/2 and blocks IL-12 production (17). In similar vein, p38 MAPK signaling in DC up-regulates IL-10 expression and induces tolerance in a mouse model of melanoma, resulting in suppression of anti-tumor T cell response, whereas inhibition of p38 signaling restored the ability of DC to stimulate T cell responses (18). The observation that p38 inhibition or MEK/ERK activation restores DC function in myeloma patients provides further evidence that p38 blockade may be of therapeutic benefit (19).

With respect to the balance between Treg versus Th17 immunity, studies in mice have shown that p38 inhibition attenuates Treg induction by DC and enhances the efficacy of DC vaccination and anti-tumor immunity (20), whereas blockade of the ERK pathway suppresses DC-driven Th17 responses (21). Collectively, these results suggest that preferential signaling though the ERK pathway may favor a switch from DC induction of Treg responses to Th17 differentiation and expansion.

In humans, treatment of ovarian tumor antigen-loaded, cytokine-matured DC with a combination of IL-15 and a p38 MAPK inhibitor offers potent synergy in antagonism of Treg induction and redirection toward Th17 responses that correlate with strong $CD8^+$ CTL activation (22). Tumor antigen-specific $CD4^+$ T cells secreted high levels of IL-17 and showed reduced expression of CTLA-4, PD-1, and Foxp3 following activation with IL-15/p38 inhibitor-treated DC. It was further shown that modulation of p38 MAPK signaling was associated with reduced expression of PD-L1 (B7-H1), loss of IDO activity, and increased phosphorylation of ERK1/2 MAPK. These observations afford an opportunity to develop innovative DC vaccination strategies to boost Th17 immunity in ovarian cancer patients.

IDO AND THE BALANCE OF POWER BETWEEN TREG AND TH17 IMMUNITY

Several lines of investigation have pointed to a pivotal role for IDO in directing Treg or Th17 responses in tumor immunity. In humans, IDO-expressing mature DC induce proliferation of $CD4^+CD25^+Foxp3^+$ Treg (9) and parallel studies in mice have shown that IDO activates Treg and inhibits their conversion to Th17-like T cells (8, 23). The pathways involved in control of Treg/Th17 differentiation by IDO have hitherto been obscure, but recent studies have revealed a relationship between IDO function and the aryl hydrocarbon receptor (AhR) on DC and T cells. Binding of the AhR promotes the generation of Treg (24–26) and AhR ligand-specific interactions may control the balance between Treg and Th17 differentiation (27, 28). Remarkably, the tryptophan catabolite kynurenine produced by IDO is a natural ligand for AhR (29, 30), thus creating a mechanism by which IDO promotes generation of Treg.

The AhR is also expressed by DC, and is required for induction of IDO expression, thus creating a feedback loop via kynurenine that maintains DC regulatory function (31). DC from $AhR^{-/-}$ mice lacked IDO and inhibited Treg development and promoted Th17 generation from naïve T cells. Addition of exogenous kynurenine restored the generation of $Foxp3^+$ Treg and diminished Th17 differentiation, reinforcing the hypothesis that IDO, kynurenine, and AhR regulate the balance between Treg and Th17 immunity.

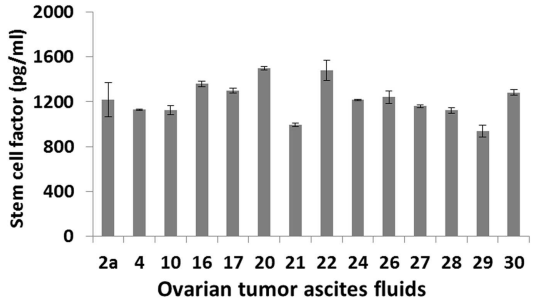

FIGURE 1 | SCF levels in primary ovarian tumor ascites fluids. Samples from 14 patients with a confirmed diagnosis of epithelial ovarian cancer were tested by ELISA.

The mechanisms by which IDO expression is regulated in ovarian tumors and tumor-associated myeloid cells are largely unknown. One potential mechanism is that IDO activity may be driven by c-KIT signaling following binding of stem cell factor (SCF), which is secreted by ovarian tumors (32–34). This proposal is based in part on recent studies showing that IDO expression can be blocked by inhibitors of c-KIT or mTOR (downstream of the c-KIT-PI3K-AKT pathway), with resultant potentiation of anti-tumor T cell responses (35). Furthermore, siRNA knockdown of SCF or blockade of c-KIT can inhibit myeloid-derived suppressor cell expansion, Treg development and tumor angiogenesis, producing a synergistic therapeutic effect in combination with immunotherapy (36). Given that SCF is abundantly present in ovarian tumor ascites (**Figure 1**), these findings raise the possibility that similar mechanisms of immune regulation may prevail in ovarian cancer.

Collectively, these observations allow formulation of an innovative model in which SCF binds c-KIT expressed by ovarian tumor cells or infiltrating myeloid cells, resulting in IDO expression. Kynurenine produced by IDO activity binds AhR on T cells and induces Treg differentiation (**Figure 2**). Drugs that block c-KIT signaling or IDO function may inhibit Treg recruitment and alleviate immune suppression by shifting the balance in favor of Th17 T cell differentiation and expansion.

INNOVATIVE STRATEGIES FOR ALLEVIATION OF IDO-MEDIATED IMMUNE SUPPRESSION

Although the ability to manipulate the functional plasticity of DC to stimulate Th17 responses against ovarian tumor antigens represents a therapeutic opportunity, DC vaccination may not be clinically effective in the face of substantial barriers imposed by immune suppression in the tumor micro-environment. Direct depletion of tumor-associated Treg has often been the favored means of alleviating immune suppression in support of DC vaccination or other tumor vaccine strategies. Depletion of Treg activity may be achieved by treatment with low dose cyclophosphamide or denileukin diftitox (ONTAK) (37, 38). The ability of cyclophosphamide to inhibit Treg and boost anti-tumor immunity has been known for decades (39), and this drug is now widely used as an

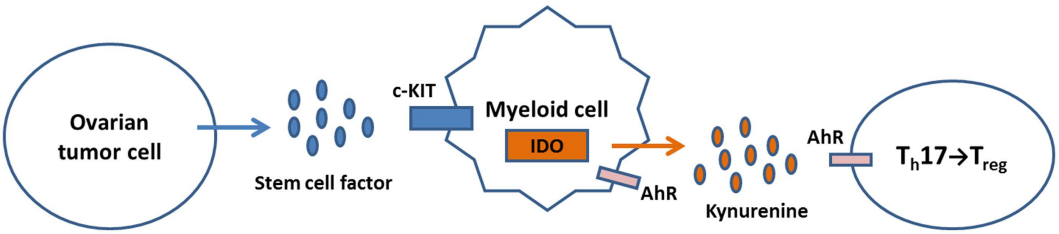

FIGURE 2 | A model for regulation of IDO expression and Treg/Th17 immunity in the ovarian tumor micro-environment. Tumor production of SCF activates c-KIT signaling in TAM, leading to induction of IDO expression. The tryptophan catabolite kynurenine binds AhR on T cells, which shifts the Th17/Treg balance in favor of Treg generation. Kynurenine can also bind AhR on myeloid cells, further inducing IDO expression through a feedback loop.

adjuvant to tumor vaccination in clinical trials. Recent studies have shown that cyclophosphamide promotes Th17 responses in cancer patients (40), lending further credence to its use as an adjuvant for DC vaccination against ovarian cancer.

A more favorable and durable approach may be to alleviate tumor-associated immune suppression through modulation of myeloid cell function and/or inhibition of IDO. If we accept the proposed model for regulation of IDO expression and Treg generation as a working hypothesis, we can infer that agents with the ability to inhibit c-KIT/PI3K/AKT/mTOR signaling or otherwise modify myeloid cell function may reduce IDO activity and inhibit recruitment of Treg in the ovarian tumor micro-environment. Our focus will be on drugs that are FDA-approved for other indications and have shown the ability to alleviate immune suppression and/or boost the efficacy of tumor vaccines or immune therapy in animal models. Promising candidates include imatinib mesylate, sunitinib, temsirolimus, and zoledronate, all of which have significant potential as adjuvants for DC vaccination against ovarian cancer.

Imatinib mesylate (Gleevec) binds BCR-ABL and c-KIT, and is an effective treatment for BCR-ABL$^+$ chronic myeloid leukemia. More recent studies have shown that the therapeutic effect of imatinib could also be attributed to immune response, overcoming tumor-associated T cell tolerance and boosting vaccine efficacy (41). Imatinib also decreased Treg frequencies and enhanced anti-tumor immune responses to DC vaccination against imatinib-resistant BCR-ABL-negative lymphoma (42), and was subsequently shown to activate CD8$^+$ T cells and induce Treg apoptosis in a gastrointestinal tumor model through c-KIT inhibition and diminished IDO expression (35).

With respect to ovarian cancer, KIT ligand (SCF) is anti-apoptotic and increases cisplatin resistance, whereas imatinib induces apoptosis (43). Although imatinib has shown minimal clinical benefit as a single agent in ovarian cancer (44, 45), it is well tolerated, and its ability to inhibit c-KIT and block IDO expression (35) suggests imatinib has potential to alleviate immune suppression as an adjuvant treatment for DC vaccination.

Sunitinib is an inhibitor of VEGFR, PDGFR, c-KIT, and Flt-3, and is FDA-approved for metastatic renal cell cancer. Sunitinib is currently being tested in over 300 clinical trials for cancer treatment (46), including ovarian cancer (47, 48). Numerous studies have shown that sunitinib can reduce myeloid suppressor cell accumulation and decrease Treg frequencies in animal models (49, 50) and in renal cell carcinoma patients (51, 52). This activity may at least in part be mediated through c-KIT and/or STAT3 signaling (49, 50). Sunitinib has been tested in combination with DC loaded with autologous total tumor RNA in a recently completed phase II clinical trial (NCT00678119), and a new phase III trial of DC vaccination for renal cell carcinoma following first-line treatment with sunitinib has recently been initiated (NCT01582672). No results have been reported to date for either trial.

Axitinib, a related tyrosine kinase inhibitor that blocks multiple targets, including c-KIT, may also have potential as adjuvant therapy for DC vaccination. Axitinib was approved by the FDA in 2012, as a second line treatment for advanced renal cell carcinoma.

Rapamycin (an mTOR inhibitor) is well known for its ability to suppress T cell responses, but it also has potential as an anticancer agent through inhibition of HIF-1, HIF-2, and VEGF. mTOR is a downstream component of the KIT-PI3K-AKT pathway, and rapamycin can reproduce imatinib-mediated reduction of IDO (35). Temsirolimus (a rapamycin analog) was FDA-approved for the treatment of renal cancer in 2007, and is first-line treatment for patients with metastatic disease. Remarkably, temsirolimus can enhance the efficacy of tumor vaccines (53), suggesting that it does not share the immunosuppressive properties of rapamycin, and may have value as an adjuvant for DC vaccination.

Although they don't act as inhibitors of c-KIT or downstream signaling, amino-bisphosphonates may also have potential as adjuvant treatments, by virtue of their ability to modify myeloid cell function. Zoledronic acid is a matrix metalloprotease inhibitor that blocks myeloid-derived suppressor cell expansion (54) and induces a pro-inflammatory shift in macrophage function, favoring M1 polarization over the pro-tumor M2 phenotype. Zoledronate can reduce expression of VEGF and IL-10 and increase production of type-1 cytokines such as IFNγ (55, 56). It is not known whether amino-bisphosphonates have any influence on IDO expression.

DIRECT INHIBITION OF IDO

While it would appear reasonable to block IDO function directly, rather than use signal transduction inhibitors that might lead to off-target effects and toxicity, agents that inhibit IDO activity are few and far between. 1-Methyl tryptophan has seen extensive experimental use as a competitive blocker of IDO function (57, 58), but its clinical use has been limited. There are currently two trials of DC vaccination combined with 1-methyl

tryptophan listed by ClinicalTrials.gov: a phase I/II trial of adenovirus p53-transduced DC vaccine with 1-methyl tryptophan for treatment of recurrent/stage IV breast cancer (NCT01042535, sponsored by the National Cancer Institute), and a phase II trial of Sipuleucel-T (Provenge®, Dendreon Corp.) with 1-methyl tryptophan for treatment of metastatic castration-resistant prostate cancer (NCT01560923, sponsored by the Masonic Cancer Center, University of Minnesota).

A second agent, INCB24360, developed by Incyte Corporation, is being tested in a phase II trial versus tamoxifen for patients with ovarian, primary peritoneal, or fallopian tube cancer and suffering biochemical recurrence, i.e., with CA125 at least twice the upper limit of normal (NCT01685255). INCB24360 is also being tested in a randomized phase II trial of ipilimumab plus INCB24360 or placebo for metastatic melanoma (NCT01604889). Should INCB24360 demonstrate efficacy in inhibition of IDO activity in the clinical setting, it may have considerable value as an adjuvant treatment for DC vaccination against ovarian cancer and other malignancies.

CONCLUSION

Elucidation of the multiple facets of immune regulation in the ovarian tumor micro-environment has sharpened the appreciation that there are formidable barriers to therapeutic DC vaccination, but has also raised the prospect for mechanism-based interventions. The observations that Treg infiltration or Th17 infiltration respectively correlate with either diminished or improved overall survival strongly indicate that blockade of Treg activity or stimulation of Th17 responses could similarly result in improved clinical outcomes. DC vaccine strategies that bias tumor-specific T cell responses toward a Th17 phenotype should be tested in clinical trials, preferably in advanced stage ovarian cancer patients that have completed surgery and chemotherapy and have minimal disease at the time of DC vaccination. The goal should be to prevent disease recurrence or progression, rather than to use DC vaccination as a salvage therapy in patients with significant tumor burden.

Although DC vaccination designed to boost Th17 immunity represents a step forward, adjuvant treatments that alleviate tumor-associated immune suppression are probably essential for any prospect of clinical success. IDO expression by ovarian tumor cells or infiltrating myeloid cells arguably forms one of the cornerstones of immune regulation in ovarian cancer, and it is no surprise that high IDO expression is associated with diminished overall survival. Drugs that block IDO expression or activity may tip the Treg/Th17 balance in favor of anti-tumor immunity, and several intriguing possibilities that are either FDA-approved for other indications or are currently in clinical trials have been considered. Targeting of the IDO/kynurenine/AhR regulatory pathway may also be an innovative approach, e.g., through the use of AhR antagonists such as resveratrol.

Other treatments that can have an impact on immune regulation in ovarian cancer should also be considered, either as stand-alone therapy or in combination with DC vaccination. Ipilimumab targets the T cell inhibitory molecule CTLA-4, which is highly expressed by Treg, and is FDA-approved for treatment of metastatic melanoma. There are many clinical trials in progress for ipilimumab in treatment of other malignancies, including ovarian cancer, but results have been variable and often discouraging. The prospects might be better for anti-PD-1 antibodies or anti-PD-L1 antibodies, for which promising results have been reported from clinical trials. Given that PD-L1 (B7-H1) expression is associated with decreased overall survival in ovarian cancer, blockade of PD-L1, or PD-1 may be an attractive option. Collectively, though, it is our opinion that the weight of evidence points to IDO as the focal target for immunological intervention in support of DC vaccination against ovarian cancer.

AUTHOR CONTRIBUTIONS

Hannah E. Goyne and Martin J. Cannon contributed equally to the preparation and writing of this manuscript.

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Dendritic cell plasticity in tumor-conditioned skin: CD14$^+$ cells at the cross-roads of immune activation and suppression

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Tumors abuse myeloid plasticity to re-direct dendritic cell (DC) differentiation from T cell stimulatory subsets to immune-suppressive subsets that can interfere with anti-tumor immunity. Lined by a dense network of easily accessible DC the skin is a preferred site for the delivery of DC-targeted vaccines. Various groups have recently been focusing on functional aspects of DC subsets in the skin and how these may be affected by tumor-derived suppressive factors. IL-6, Prostaglandin-E2, and IL-10 were identified as factors in cultures of primary human tumors responsible for the inhibited development and activation of skin DC as well as monocyte-derived DC. IL-10 was found to be uniquely able to convert fully developed DC to immature macrophage-like cells with functional M2 characteristics in a physiologically highly relevant skin explant model in which the phenotypic and functional traits of "crawl-out" DC were studied. Mostly from mouse studies, the JAK2/STAT3 signaling pathway has emerged as a "master switch" of tumor-induced immune suppression. Our lab has additionally identified p38-MAPK as an important signaling element in human DC suppression, and recently validated it as such in *ex vivo* cultures of single-cell suspensions from melanoma metastases. Through the identification of molecular mechanisms and signaling events that drive myeloid immune suppression in human tumors, more effective DC-targeted cancer vaccines may be designed.

Keywords: dendritic cells, human DC subsets, skin, macrophages, cancer, immune suppression

DENDRITIC CELL SUBSETS AND THEIR PLASTICITY IN HUMAN SKIN: IMPACT ON CANCER VACCINATION

Skin is the largest human organ and its direct contact with the outside environment requires tightly regulated surveillance mechanisms to keep potentially harmful intruders at bay. For this purpose, human skin is densely populated with patrolling myeloid cells, such as Langerhans cells (LC) in the epidermal outer layer and various dermal dendritic cell (DDC) subsets and macrophages in the dermal layer (1, 2). It has been elegantly shown that different profiles of pattern recognition receptors present on the various myeloid subsets lining the skin makes them exquisitely specific in the recognition, uptake and either direct elimination of pathogenic microbes, or in presentation of pathogen-associated antigens for subsequent activation of the adaptive immune system (3–5). Interaction of a pathogen with pathogen-recognition receptors on dendritic cell (DC) induces activation of down-stream signaling pathways that result in their enhanced ability to process and present pathogenic antigens and in their migration to the draining lymph nodes, accompanied by phenotypic and morphological maturation, and priming of antigen-specific T or B lymphocytes (6). Whereas initially studies concerning DC subsets in human skin mostly involved the most predominant subsets, i.e., CD1a^{hi}Langerin$^+$ LC, CD1a$^+$ DDC, and CD14$^+$CD1a$^-$DDC (7–9), the characterization of new surface markers and deeper phenotypic and functional analyses now show that further distinctions can be made (10–13).

From our own work and that of others, it has become clear that beside epidermal LC and dermal macrophages at least five migratory DDC subsets can be distinguished (13, 14), i.e., CD1a$^+$CD14$^-$ DDC, CD1a$^+$CD14$^+$ DDC, CD1a$^-$CD14$^+$ DDC, and two double-negative subsets. An important issue that as yet remains unresolved is whether all these DC populations represent genuine subsets, or whether they are part of the same DC subset in various states of activation or differentiation. A growing number of studies now point to the existence of an inter-related population of cutaneous DC and macrophages in flux, trans-differentiating into each other as directed by environmental cues (8, 15, 16). This has direct consequences for the type of immune responses that will ensue, as different migratory DC sub-populations have now been directly linked to the induction of different types of immunity (13, 14) and have different capacities to cross-present antigens for the activation of cytotoxic CD8 T cells, a process crucial for the induction of anti-tumor immunity. Roughly, CD1a$^+$ mature LC and DDC subsets have been linked to type-1 T cell mediated immunity, whereas CD14$^+$ immature DDC subsets have been linked to the induction of humoral immunity and expansion of regulatory T cells (Treg) (11, 12); see **Figure 1** for a schematic overview. Recent evidence suggests that tumors like melanoma abuse the balance between

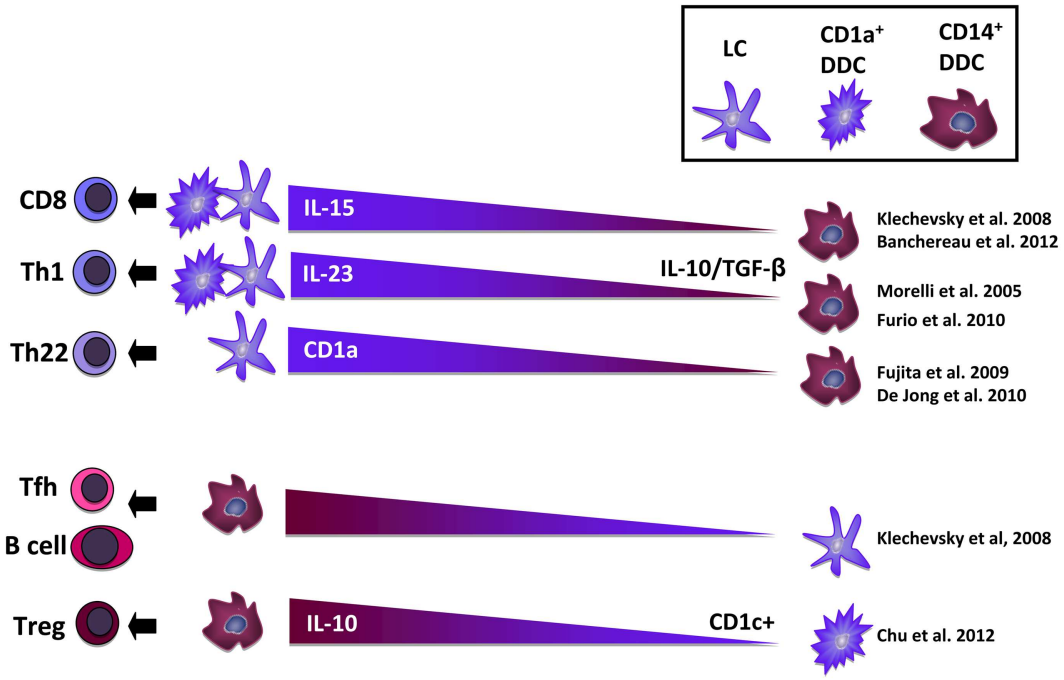

FIGURE 1 | Overview of the reported T cell differentiation induction abilities of mature Langerhans cells (LC) and CD1a$^+$ dermal dendritic cells (DDC) vs. immature CD14$^+$ DDC. Abbreviations: DDC, dermal dendritic cell; IL, interleukin; LC, Langerhans cell; Th, T helper cell; Tfh, T follicular helper cell; Treg, T regulatory cell; TGF-β, transforming growth factor – β.

these subsets to effectively escape immune recognition (13, 17). In order for DC-targeted vaccines delivered through the skin to be effective, tumor-induced immune suppression should be overcome and T cell-stimulatory DC subsets selectively targeted. Here, we discuss mechanisms of tumor-imposed DC suppression in the skin microenvironment and how these may be counter-acted in aid of DC-based immunotherapy.

LC AND CD1a$^+$ DDC: T CELL ACTIVATION

Klechevsky et al. first described a functional dichotomy between human LC and CD14$^+$ DDC with the former preferentially activating CD8$^+$ T cells and the latter B cells (9). In recent publications primary human LC have been shown to be superior inducers of Th22 cells (including conventional variant αβ-T cells restricted through CD1a) (18, 19). IL-22 has an important barrier function in homeostasis and safeguards the integrity of epithelial layers, but is also involved in pathological skin conditions like psoriasis. Furio et al. reported a superior ability of migratory LC over DDC to induce either Th1 or Th2 responses (20). Of note, DDC in this report consisted of CD1a$^-$CD14$^-$ double-negative DDC with a potentially lower capacity for T cell activation than CD1a$^+$ DDC. Mathers and co-workers showed that while LC were superior Th17 inducers, human CD14$^-$ DDC had the ability to skew Th cells to either a Th1, Th2, or Th17 profile, depending on their environmental conditioning, number, and activation state (21). To further delineate T cell-stimulatory properties of freshly isolated human LC vs. CD1a$^+$ DDC, we undertook a genome-wide transcriptional profiling analysis which revealed CD1a$^+$ DDC to express a far wider range of adhesion and co-stimulatory molecules, chemokines, and cytokines (and at higher levels), pointing to a putatively superior migratory and T cell stimulatory ability over LC in steady state conditions (22). Using a human cell line model of LC and CD1a$^+$ DDC differentiation, we confirmed these data and showed DDC to be superior activators of cytotoxic CD8$^+$ T cells. Importantly, this was validated in the same study by a comparative assessment of the *ex vivo* ability of human skin-emigrated LC vs. DDC subsets to prime HLA-A2-matched CD8$^+$ T cells against an epitope derived from the MART-1 melanoma antigen (23). While LC and CD1a$^+$ DDC were equally effective in priming allogeneic Th cells, DDC primed significantly higher rates of MART-1 recognizing CD8$^+$ T cells at a higher functional avidity. Of note, Banchereau et al. have recently linked the superior effector CD8$^+$ T cell priming capacity of LC and CD1a$^+$ DDC to their release of IL-15 into the immunological synapse (12).

CD14$^+$ DDC: T CELL TOLERIZATION

CD14$^+$ migratory DDC are discernable from dermis-resident CD14$^+$ dermal macrophages through their surface expression of CD1b and CD1c (24). In a comparative analysis with CD14$^-$ DDC, CD14$^+$ DDC were shown to be poor inducers of allogeneic T cells and to require high DC:T cell ratios for Th1 induction (25). This relative inability of CD14$^+$ DDC to induce Th1 cells was related to their release of IL-10 and TGFβ1. We and others have found CD14$^+$ DC to carry low levels of co-stimulatory molecules, to display a poor T cell priming capacity, and to be characterized by the expression of CD141/BDCA3 (Thrombomodulin), a marker that

has been linked to a human DC subset with cross-priming ability (11, 13, 26). These $CD14^+BDCA3^+$ migratory DDC in a report by Chu et al. were shown to constitutively release IL-10 and to induce T cell hyporesponsiveness and Tregs (11). Moreover, they were able to cross-present self-antigens and inhibit skin inflammation in an *in vivo* transplantation model. These data point to an important role for this subset in T cell homeostasis. Banchereau et al. have pin-pointed the inability of $CD14^+$ DDC to prime effector $CD8^+$ T cells to their release of IL-10 and TGFβ (12) and the expression of Ig-like transcript 4 (ILT4) and ILT2 (27).

TUMORS ABUSE DC PLASTICITY TO UNDERMINE IMMUNITY: A CENTRAL ROLE FOR $CD14^+$ DC

A large number of studies attest to the remarkable plasticity of the myeloid lineage; tumors abuse this phenotypic plasticity to re-direct myeloid differentiation toward the development of immune-suppressive subsets that effectively interfere with anti-tumor immunity (28). Consequently, tumors are often characterized by an infiltrate of immature macrophage-like cells and a lack of infiltrating DCs, which is generally a poor prognostic sign (28). We and others have shown that DC differentiation from monocytes can be blocked by tumor-derived soluble factors (most notably IL-10, IL-6, or PGE2) resulting in the development of $CD14^+$ macrophage-like cells with poor T cell stimulatory abilities (so-called M2-type macrophages) and with T cell suppressive activity (**Figure 2**) (29–32). Beside monocytes, fully differentiated DC can be recruited to the tumor microenvironment, where they may lose their characteristic CD1a expression through the suppressive action of IL-10, as shown for melanoma metastases (33). A growing number of studies indicates the unique ability of tumor-associated IL-10 to convert even fully differentiated DC to $CD14^+$ suppressive macrophage-like cells (8, 15, 16, 34, 35). IL-10 is generally expressed at high levels in the microenvironment of metastatic melanoma and can either be directly derived from tumor cells or from infiltrating immune cells. Among a panel of tumor-associated suppressive factors, we found IL-10 uniquely able to convert DCs to immature macrophage-like cells in two human model systems: (1) a physiologically highly relevant skin explant model in which we studied the phenotypic and functional traits of "crawl-out" myeloid cells (13) and (2) an *in vitro* model of tumor-conditioned DC maturation in which we functionally assessed $CD14^-$ and $CD14^+$ DC that had developed from monocyte-derived DC (MoDC) during IL-10-exposed maturation (17). In all above mentioned cases the tumor-induced M2-like cells shared some striking traits: an immature $CD14^+BDCA3^+DC\text{-}SIGN^+CD16^+$ phenotype and macrophage-like morphology (**Figure 2**), a disturbed balance in the release of immunosuppressive IL-10 (high) vs. immunostimulatory IL-12p70 (low), high expression levels of the T cell-inhibitory molecule B7-H1/PDL-1, and lower priming efficiency of allogeneic Th cells and of $CD8^+$ (killer) T cells, the latter specifically recognizing the melanoma antigen MART-1, but binding epitope/MHC complexes with low avidity (13, 17, 32, 35).

In studies assessing CD1a and CD14 expression on DC from human skin explants, we showed the intracutaneous cytokine balance to be important for the subset composition of migrated DC (8, 13). Indeed, we have found compelling evidence that LC and $CD1a^+$ DDC can actually trans-differentiate during and after

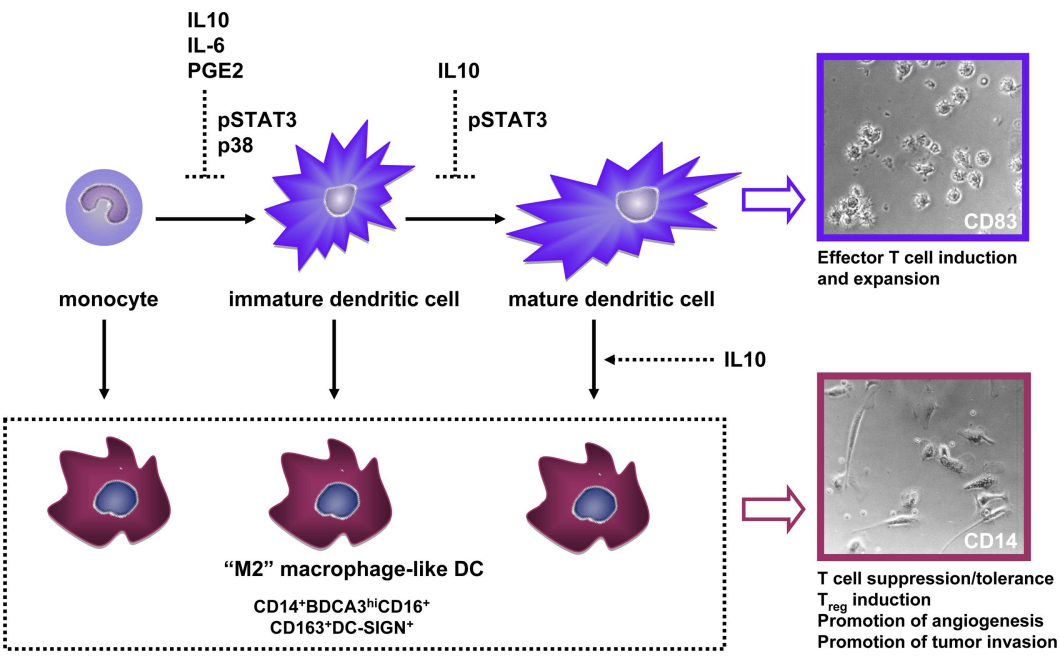

FIGURE 2 | Interference by tumor-associated soluble factors with normal dendritic cell (DC) development through indicated underlying signaling pathways, leads to (trans-)differentiation of $CD14^+$ M2-macrophage-like cells with immune-suppressive and tumor growth- and invasion-promoting properties. Photographic inserts illustrate the DC and adherent macrophage-like morphology of human skin-emigrated $CD83^+$ and $CD14^+$ DC, respectively (magnification 400×). Abbreviations: IL, interleukin; PGE2, prostaglandin-E2.

migration from human skin explants to a $CD14^+$ macrophage-like state in an IL-10-dependent fashion. Dermal conditioning by IL-10 or by topical application of irritants resulted in a shift among migrated DC from a mature $CD83^+CD1a^+$ state to an immature $CD83^-CD14^+$ macrophage-like state, passing through a $CD1a^+CD14^+$ intermediate stage (8, 15). Based on the fact that these $CD14^+$ cells also expressed CD1c they were classified as DC rather than macrophages. Moreover, topical application of irritants to epidermal sheets showed that trans-differentiation from LC to macrophage-like DC depended on the presence of dermal fibroblasts and could be blocked by IL-10 neutralizing antibodies (15). A similar observation has been described in mice, where the presence of a subcutaneous tumor resulted in a DC-to-macrophage shift, with macrophage-like cells producing immune-suppressive factors such as IL-10, iNOS, and Arginase (16). Importantly, this trans-differentiation among DC that had migrated from human skin was preventable by co-injection of the DC-activating cytokines GM-CSF and/or IL-4 prior to skin explant culture (8).

Consistent with their expression of the M2-macrophage marker CD163, IL-10-converted $CD14^+$ cells induced IL-10 and FoxP3 mRNA expression in allogeneic Th cells as well as a Th2-like cytokine profile and Treg expansion (13). Consistent with these tolerogenic qualities, IL-10-induced $CD14^+$ macrophage-like MoDCs expressed high levels of immune suppression-related transcripts such as Indoleamine 2,3-dioxygenase (IDO), IL-4Rα, IL-6R, TGFβ1, HIF1α, and VEGFA (17). Activation of a HIF1α transcriptional signature has been reported in tumor-associated macrophages, even under normoxic conditions (36). This is in line with the transcriptional and cytokine release profiles we observed for $CD14^+$ IL-10-conditioned DC, which revealed coordinated expression of HIF1α, TGFβ, VEGFA, MMP3, MMP9, IL-8, and TNFα, all of which can contribute to such tumor-promoting processes as endothelial cell migration and proliferation and tumor growth and invasion (28). In conclusion, tumor-related suppressive factors can divert DC during differentiation and even during and after maturation toward a macrophage-like state with immune-suppressive and pro-angiogenic and pro-tumor invasive properties (**Figure 2**).

Interestingly, in DC migrating from human skin, BDCA3 and DC-SIGN expression levels showed a very significant inverse correlation with CD83 maturation marker expression, indicating the utility of these markers for the identification of immature DC. Indeed, they marked $CD14^+$ skin-emigrated DC as the least mature population with poor co-stimulatory properties (13). In keeping with these observations, DC that had migrated from skin explants taken from breast cancer mastectomy specimens, predominantly consisted of the $CD14^+$ DC subset with a macrophage-like morphology (13). Normalized distribution (i.e., more mature and less immature DC subsets) was observed for explants taken from patients that had received neoadjuvant chemotherapy: a clear indication that prevailing migration of the immature $CD14^+$ subset was tumor-related.

From our observations we conclude that combined expression of CD14, BDCA3, DC-SIGN, CD16, and CD163 provides a phenotypic profile useful for the identification of M2-macrophage-like subsets with immune-suppressive and tumor-promoting characteristics that arise during tumor-conditioned differentiation or maturation of human DCs. We and others have found evidence of phenotypically similar subsets in breast, colon, head and neck, renal cell, and melanoma tumors (17, 37–39). Indeed, in single-cell suspensions derived from a panel of six metastatic melanoma tumors, we observed by multicolor flow cytometry analysis, that $CD14^+$ cells, co-expressing both DC-SIGN and BDCA3 and detectable in a range of 1–38%, significantly outnumbered $CD1a^+$ DC, which were virtually absent (ranging from 0.05 to 0.1%) (17). BDCA3 expression has recently been reported on skin-derived $CD14^+$ DC that induced inflammation-attenuating Tregs (11). Combined with its association with cross-presenting DC subsets (10), this is highly suggestive of cross-tolerizing ability for $BDCA3^+$DC. As yet, the functional significance of BDCA3/CD141 in either cross-presentation or immune suppression remains largely unclear, but some clues are emerging. Its Lectin-like domain can down-regulate NF-κB and mitogen-activated protein kinase (MAPK) pathways and might thus interfere with DC maturation and drive IL-10 release and Th2 skewing (40, 41). In keeping with this notion, $BDCA3^+$ blood DC promote Th2 skewing (42) and *in vitro* generated or skin-derived $CD14^+BDCA3^+$ DC release elevated levels of IL-10 (11, 34). In addition, DC-SIGN can negatively impact DC activation resulting in prolonged and increased IL-10 transcription (43). Both DC-SIGN and BDCA3 may thus contribute to the immune-suppressive activity of tumor-modulated $CD14^+$ cells.

Recently, the role of non-coding RNAs or microRNAs (miRNAs) in myeloid cell plasticity and functionality has also been studied. In mice, tumor-associated miRNAs were found to modulate the survival and longevity of DC (44), miR-223 was described to negatively regulate and miR-150 to positively regulate the cross-presenting abilities of LC (45, 46), the TGF-β associated miR-27a was reported to inhibit DC-mediated differentiation of Th1 and Th17 cells (47) and in an allergy setting miR-23b was shown to induce tolerogenic DC through inhibition of the Notch1/NF-κB pathway (48). In man, this field of research remains largely unexplored, though miR-155 was shown to regulate the M1/M2-macrophage balance by targeting the IL13-Receptor α1, thereby reducing M2 polarization (49).

SIGNAL TRANSDUCTION PATHWAYS ACTING AS MASTER SWITCHES OF TUMOR-INDUCED DC SUPPRESSION: TARGETS FOR THERAPEUTIC INTERVENTION

Tumor-derived suppressive factors bind various receptors on myeloid cells but down-stream signals may converge in shared pathways. Mostly from mouse studies, the JAK2/STAT3 signaling pathway has emerged as a "master switch" of tumor-induced immune suppression (50). We have additionally identified p38-MAPK as an important signaling pathway in human DC suppression, and validated it as such in *in vitro* DC cultures and in *ex vivo* cultures of single-cell suspensions from melanoma metastases (32). From a panel of tumor-associated suppressive factors (including PGE2), we found only IL-6 and IL-10 to induce STAT3 phosphorylation during human MoDC development. As we had previously identified prostaglandins as the main culprit of suppressed DC differentiation by supernatants from single-cell suspensions of metastatic melanoma tumors (29) it was not

surprising that STAT3 inhibition alone could not prevent this suppression; for this, combined JAK2/STAT3 and p38-MAPK inhibition was required. Importantly, combined interference in the STAT3 and p38 pathways completely prevented inhibition of DC differentiation by all tested tumor supernatants ($n = 18$, derived from both primary tumors and tumor cell lines, together encompassing eight different histological origins) and led to superior DC functionality, evidenced by increased allogeneic T cell reactivity with elevated IL-12p70/IL-10 ratios and Th1 skewing (32). Most importantly, combined STAT3 and p38 inhibition supported a shift from $CD14^+$ monocyte-like cells to $CD1a^+$ DC in metastatic melanoma single-cell suspensions, indicating a potential for improved DC differentiation in the tumor microenvironment (32). Of note, siRNA-mediated knockdown of STAT3 only, did effectively prevent the generation of $CD14^+$ cells during IL-10-modulated MoDC maturation induction (17).

Altogether, these data point to different tumor-associated factors (i.e., IL-10, IL-6, PGE2) exerting their suppressive effects at various stages of myeloid DC development through converging and communicating signaling elements encompassing the JAK2/STAT3 and p38-MAPK pathways (**Figure 2**). To specifically address melanoma-induced myeloid suppression it is important to further dissect the JAK2/STAT3 and p38-MAPK pathways and possible cross-talk between them in melanoma-associated myeloid subsets in order to identify specifically acting and clinically relevant therapeutic targets. The advent of small-molecule kinase inhibitors and RNAi-based therapeutics now enables targeting not only of tumors, but also of their stroma, and should facilitate re-programing of tumor-associated myeloid cells, as well as tumor-modulated DC subsets in the skin, in support of anti-tumor immunity.

THERAPEUTIC ACTIVATION AND TARGETING OF DC IN THE SKIN AND ITS LYMPH CATCHMENT AREA

Beyond the local suppressive environment at the site of the tumor, the immune-suppressive effects of the tumor stretch to draining lymph nodes where anti-tumor T cell responses should be primed. Sentinel lymph nodes (SLN) are the first-line tumor-draining lymph nodes and as such bear the brunt of melanoma-induced immune suppression (51). We have identified and characterized four conventional DC subsets in melanoma SLN, two of which were positively identified as skin-derived $CD1a^+$LC and DDC, and the remaining two ($CD1a^-CD14^-$ and $CD1a^-CD14^+$) as LN-resident subsets with varying levels of BDCA3 and DC-SIGN expression (52). Deeper invasion of the primary melanoma in SLN tumor negative patients was related to a reduced activation state of skin-derived DC subsets in the SLN (53, 54). Also, lower frequencies of the skin-derived subsets were found in tumor positive SLN as well as a reduced activation state of LN-resident DC subsets (our own unpublished data). These findings indicate a local suppressive effect of the primary tumor on the activation state of skin-derived DC which then migrate to the SLN and lymph node metastasis-related suppression of SLN-resident DC subsets, and are in keeping with tumor-induced conditioning of the microenvironment (skin or SLN, respectively). Moreover, they suggest that primary melanoma-mediated suppression of activation and migration of skin DC enables local metastasis.

In two Phase II clinical trials we have demonstrated that localized intradermal administration of DC-stimulatory agents such as GM-CSF and CpG oligodeoxynucleotides (ODN), i.e., TLR9 ligands, led to increased activation of DC subsets in SLN of melanoma patients and tipped the local cytokine balance in favor of cytotoxic T cell mediated anti-tumor immunity (55–58). Although in man CpG ODN don't directly bind to conventional DC, we nevertheless observed maturation induction of conventional DC subsets, most likely through CpG-induced cytokine release by plasmacytoid DC (57). In our human skin explant model we have similarly tested the effects of intradermal delivery of a panel of TLR-ligands on migratory DC and found a unique ability of the TLR2 and 3 agonists peptidoglycan (PGN) and polyriboinosinic-polyribocytidylic acid (Poly I:C) to enhance the T cell-priming ability of skin-emigrated DC, which, in the case of PGN, was accompanied by Th1 polarization (59). Surprisingly only small effects of the tested TLR-ligands on phenotypic DC activation were observed. This may have been due to induced IL-10 release, which might have been counter-acted by simultaneous signaling modulation (60, 61). Indeed, evidence for the therapeutic efficacy of combined STAT3 inhibition and CpG ODN was previously provided by Kortylewski and colleagues, showing superior immune stimulatory effects of CpG by eliminating collateral STAT3-mediated suppressive effects (62, 63).

In conclusion, JAK2/STAT3 and/or p38-MAPK signaling interference, combined with local immune potentiation, may counterbalance tumor-imposed suppression of skin DC subsets, minimizing the induction and trans-differentiation of migratory $CD14^+$ M2-like DC with T cell suppressive characteristics, and thus set the stage for effective tumor vaccination through DC-targeted approaches.

AUTHOR CONTRIBUTIONS

Rieneke van de Ven and Tanja D. de Gruijl conceptualized and wrote the manuscript, Jelle J. Lindenberg and Dinja Oosterhoff co-wrote the manuscript.

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The role of dendritic cells in graft-versus-tumor effect

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Dendritic cells (DCs) are the most potent antigen presenting cells. DCs play a pivotal role in determining the character and magnitude of immune responses to tumors. Host and donor hematopoietic-derived DCs play a critical role in the development of graft-versus-host disease (GVHD) following allogeneic hematopoietic cell transplantation. GVHD is tightly linked with the graft-versus-tumor (GVT) effect. Although both host and donor DCs are important regulators of GVHD, the role of DCs in GVT is poorly understood. GVT is caused by donor T cells that attack recipient tumor cells. The donor T cells recognize alloantigens, and tumor specific antigens (TSAs) are mediating GVHD. The process of presentation of these antigens, especially TSAs remains unknown. Recent data suggested that DC may be essential role for inducing GVT. The mechanisms that DCs possess may include direct presentation, cross-presentation, cross-dressing. The role they play in GVT will be reviewed.

Keywords: allogeneic hematopoietic stem cell transplantation, graft-versus-tumor effect, dendritic cells

INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) has become widely used as a curative therapy for a variety of life-threatening hematologic, immunologic, and genetic diseases. However, serious complications endure, presenting as obstacles to successful treatment. One complication is graft-versus-host disease (GVHD) and another is primary disease relapse. The current understanding of the science suggests that the dysregulation and/or dysfunction of the immune system and corresponding immunocompetent cells of recipients after allo-HCT are responsible for these obstacles (1). Current prophylaxis and treatment regimens using immunosuppressants mainly target T cells for the mitigation of GVHD. Excessive immunosuppression for the treatment of GVHD often results in serious infections (*Cytomegalovirus*, Herpes zoster virus, fungus, and bacterias), decreases graft-versus-tumor (GVT) responses (which are the most beneficial effects of allo-HCT), and finally is known to cause relapse of primary disease (1, 2). Thus, it is imperative that we develop new strategies of GVHD prophylaxis and treatment while maintaining sufficient GVT effect.

Dendritic cells (DCs), the most potent of the antigen presenting cells (APCs) of both the innate and adaptive immune responses, are critical for the pathophysiology of both GVHD and GVL (1–3). Host and donor hematopoietic-derived APCs (particularly DCs) are critical in the development of GVHD (4–6). In addition, host hematopoietic-derived DCs also play a significant role in GVL (7, 8). In this review, we focus on the role of DCs in GVT and consider strategies for effective utilization in enhancing GVT.

SUBSETS AND FUNCTION OF DENDRITIC CELLS

Dendritic cells have bilateral characteristics, as DCs are critical for priming T cell responses in an inflammatory milieu, but are also required for the induction of tolerance at steady state.

Dendritic cells are phenotypically classified under many subtypes. This heterogeneity suggests that better understanding of these distinct subsets may lead to the ability to modify and manipulate DC functions. Lymphoid and non-lymphoid tissues, as well as the blood, contain a variety of DC subsets with a wide range of functions. DCs arise from bone marrow (BM)-derived macrophage/DC precursors (MDPs) (9). MDPs differentiate into monocytes, yielding macrophages; common DC precursors (CDPs), which generate classic DC (cDC)-restricted precursors (pre-cDCs); or plasmacytoid DCs (pDCs) (9). However, human equivalents of mouse MDPs and CDPs remain elusive (10). Pre-cDCs migrate from the BM and enter blood circulation destined for lymphoid organs and/or peripheral tissues. Upon arrival, pre-DCs differentiate into lymphoid/non-lymphoid tissue DCs (9). DCs express both the hematopoietic marker CD45 and integrin CD11c. Further, DCs can be divided into two major categories in lymphoid tissues, based upon the intensity of CD11c expression. The first is conventional DCs (cDCs–CD11c^{high}) and second is pDCs (pDCs–CD11c$^{low/int}$). cDCs are further categorized into lymphoid organ resident DCs and migratory tissue DCs. Both categories of cDCs are also divided into CD8α^+DCs (lymphoid-derived DCs) and CD8$^+\alpha^-$DCs (myeloid-derived DCs) and they show low co-stimulatory molecules in steady state (11–13). In non-lymphoid tissue, there are three types of DCs [tissue-resident steady state DCs, pDCs, and monocyte-derived DCs (moDCs)] in mouse; humans express at least two types of DCs, pDCs, and myeloid-derived DCs that are divided into three different categories: CD16$^+$DCs, BDCA1$^+$, and BDCA3$^+$ DCs. Although DCs expressing certain phenotypes are known to contribute to development of GVHD, but not obligatory (14–16), the function of the remaining phenotypes is less understood. The various subsets are discussed very briefly below and summarized in **Table 1**, in light of several recent excellent reviews on these subsets (13, 17–19).

Table 1 | Dendritic cell subsets.

DC subsets	Surface markers	Transcription factors	Function
Mouse: CD8α$^+$DCs	Mouse: CD8α$^+$ (11)	FMS-related tyrosine kinase 3 (Flt3) (171, 172) Interferon regulatory factor 8 (IRF8) (24, 30) Inhibitor of DNA binding protein 2 (Id2) (31, 36) Basic leucine zipper transcription factor ATF-like 3 (Batf3) (32) Nuclear factor interleukin-3 regulated (Nfil3) (33) PU.1 (34) Zinc finger transcription factor (Zbtb46) (35)	Engulf and process exogenous antigens and subsequently present these antigens to CD4$^+$ T cells via MHC class II (13) Strong cross-presentation capacity (37) Enhancement of CTL responses (38, 39) Secrete large amounts of IL-12 (38, 39) Secretion of type I IFN with TLR3, TLR9, and plasmodium stimulation (173, 174) Immune modulatory function (13) Decrease allogeneic T cell proliferation (28, 40, 175) Induce FoxP3$^+$ Treg and IL-10 secreting T cells (40, 41) Induction of peripheral self-tolerance (176)
Human: BDCA3$^+$DCs	Human: BDCA3$^+$ (CD141)$^+$ (48) Mouse/human: MHC class II$^+$, CD24$^+$ (12), CD36$^+$ (24), DEC205 (CD205)$^+$ (12), Clec9A (DNGR-1)$^+$ (22), TLR3$^+$ (23), XCR$^+$ (25, 46, 47)		
CD8α$^-$DCs	Mouse: CD8α$^-$ (17), CD11b$^+$ (17), CD209 (DC-SIGN)$^+$ (51), CD172a (Sirpα)$^+$ (52), DC inhibitory receptor 2 (DCIR2)$^+$ (53), dectin-1 (Clec-7a)$^+$ (54)	FMS-related tyrosine kinase 3 (Flt3) (17), lymphotoxin β receptor (LTβR) (17), notch RPB-J (55), notch receptor 2 (57), reticuloendotheliosis homolog B (RelB) (177), TNF-associated factor 6 (TRAF6) (178)	Enhancement of Th2 responses in primary stimulation (58) IL-12 production under certain conditions (59) CD4$^+$ T cell activation (53) Cross-presentation of particular antigens under certain conditions (54, 179, 180)
Plasmacytoid DCs (pDCs)	Mouse: CD11c^{int} (18), B220 (CD45RA)^{hi} (18), sialic acid-binding immunoglobulin-like lectins-H (Siglec-H)^{hi} (18), CD317 (mPDCA-1)^{hi} (18) Human: BDCA-2$^+$ (60), BDCA-4$^+$ (60), DCIR$^+$ (61), Ly6C$^+$ (62), DC-SIGN$^+$ (63), CD123$^+$ (64)	Ikaros (68), STAT-3 (68, 181), STAT-5 (181) (182)	Secretion of type I IFNs (18, 62) Immunomodulation (18) Increased cross-presentation capacity (183)
Monocyte-derived DCs Inflammatory DCs (infDCs)	Mouse (19): MHC class II$^+$, CD11b$^+$, CD11c$^+$, F4/80$^+$, Ly6C$^+$, CD64$^+$, M-CSFR$^+$, ZBTB46$^+$ Human (184): HLADR$^+$, CD11c$^+$, BDCA1$^+$, CD1a$^+$, FcεRI$^+$, CD206$^+$, CD14$^+$, M-CSFR$^+$, ZBTB46$^+$	Unknown	Migration into the site of inflammation from BM in a CCR2-dependent manner (77) Activation and proliferation of T cells (185–188) Production of various cytokines (185–188)
Human: BDCA1DC (CD1c$^+$DCs)	BDCA1$^+$ (60), CD11c$^+$ (79), HLADR$^+$ (79), CD86$^+$ (83), CCR5$^+$ (83), FcγR$^+$ (161)	Unknown	Secretion of high levels of IL-12, following TLR4 and TLR7 stimulation (83, 161) Stimulation of allogeneic T cells (79) Increased cross-presentation capacity (46–48, 83–85)

CD8α$^+$DCs (MOUSE) AND BDCA3$^+$DCs (HUMAN)

CD8α$^+$DCs are approximately 20–40% of total mouse splenic DCs and around 70% of murine thymic DCs (11, 12). In steady state, they express low levels of co-stimulatory molecules, such as CD80, CD86, and CD40 but high levels of MHC class II (20, 21) and highly express CD24, CD36, DEC205 (CD205), Clec9A (DNGR-1), TLR3, and XCR, but show little or no expression of CD172a (Sirpα), CD11b, and DCIR2 (33D1) (12, 22–26). The administration of Flt-3L to WT mice dramatically expands CD8α$^+$DCs that are phenotypically and functionally matured (27) and have a reduced capacity for allogeneic T cell stimulation (28).

Certain transcription factors play an important role in the development of CD8α$^+$DCs (29–36). Interferon regulatory factor 8 (IRF8) (29, 30), inhibitor of DNA binding protein 2 (Id2) (31, 36), the basic leucine zipper transcription factor ATF-like 3 (Batf3) (32), nuclear factor interleukin-3 regulated (Nfil3) (33), PU.1 (34), and zinc finger transcription factor zbtb46 (35) are critical for the development of CD8α$^+$DCs. Mice lacking these transcription factors exhibit dramatically reduced numbers of CD8α$^+$DCs while absence of zbtb46, which results in increased CD8α$^+$DCs.

CD8α$^+$DCs are unique in which they can present exogenous antigens on their MHC class I molecules, a process known as cross-presentation (37). In addition, CD8α$^+$DCs are critical for

cytotoxic T cell (CTL) responses as they are the predominant producers of IL-12 (38, 39). On the other hand host-derived CD8α$^+$DCs, expanded by the administration of Flt-3L, decrease allogeneic T cell responses *in vivo* (28). We have also found that immunization of donors with host-derived CD8α$^+$DCs, reduced acute GVHD by increased secretion of IL-10 from donor-derived T cells (40). CD8α$^+$DCs can also induce Foxp3$^+$ regulatory T cells (Tregs) in a TGF-β-dependent manner *in vitro* and *in vivo* (41). Moreover, CD8α$^+$DCs are responsible for induction of peripheral self-tolerance by their ability to capture and cross-present tissue-associated antigens to naïve CTLs (42–44) or by CD8α$^+$DCs derived TNF-mediated killing (45).

Although CD8α$^+$DCs present only in mice, recent studies have identified human equivalents. BDCA3$^+$ (CD141$^+$) DCs, which express Clec9A and XCR-1 were identified as human homologs of mouse CD8α$^+$DCs (46–49). BDCA3$^+$ DCs have the ability to cross-present soluble or cell-associate antigen to CD8$^+$ T cells (47, 48). Aside from the capacity for cross-presentation, BDCA3$^+$DCs produce IFN-α after TLR3 stimulation, similar to CD8α$^+$DCs homologs in mouse (50).

CD8α$^-$DCs (CD11b$^+$DCs)

CD8α$^-$DCs (CD11b$^+$DCs) lack expression of the marker CD8α but express CD11b, which represent a large percentage of splenic or lymphoid resident DCs (17). CD8α$^-$DCs predominately express CD209 (DC-SIGN) (51), CD172a (Sirpα) (52), DC inhibitory receptor 2 (DCIR2) (53), and dectin-1 (Clec-7a) (54). Notch RBP-J, is important for development and homeostasis of CD8α$^-$DCs (55). Recent reports also suggest that Notch 2 signaling is required for the development of a subset of splenic CD11b$^+$ DCs (CD11b$^+$ESAM$^+$DCs) and intestinal CD103$^+$CD11b$^+$DCs (56), as well as terminal differentiation of CD8α$^+$DCs and CD11b$^+$DCs (57). CD8α$^-$DCs are required to enhance Th2 responses in primary stimulation (58) and also they produce IL-12 under certain conditions (59). CD8α$^-$DCs exist in the marginal zone of the splenic lymphoid follicles and take up, process, and present exogenous antigen to CD4$^+$ T cells via MHC class II (17, 53).

PLASMACYTOID DCs

Plasmacytoid DCs are distinguished in mice by the expression of CD11c^{int}, B220 (CD45RA)^{hi}, sialic acid-binding immunoglobulin-like lectins-H (Siglec-H)^{hi}, and CD317 (mPDCA-1)^{hi} (18). In human, pDCs express BDCA-2 (60), BDCA-4 (60), DCIR (61), Ly6C (62), DC-SIGN (63), or CD123 (64). Flt3-L is a critical cytokine for the expansion of pDCs (65, 66), whereas HIF-1α is a negative regulator of pDC development *in vitro* and *in vivo* (67). Ikaros and STAT-3 play a role in the development of pDCs (68). The main function of pDCs is to produce type I interferons (IFN), such as IFN-α and IFN-β, in response to viral, fungal, and bacterial antigens (18). The role of pDCs in mediating acute GVHD is distinct depending on whether they are derived from the host or donor (69, 70).

MONOCYTE-DERIVED DCs

According to recent reports, monocytes exist in the blood as terminally differentiated cells derived from MDP [whose progenitor is common myeloid precursors (CMPs) in the BM]. In an inflammatory environment, monocytes differentiate into MoDCs, or inflammatory DCs (infDCs) and subsequently migrate into the site of inflammation (71, 72). Monocytes also contribute to the development of CD103$^-$CD11b$^+$DCs in a Csf-1-dependent manner (73, 74). Mouse BM-derived DCs generated *in vitro* with GM-CSF alone or in combination with IL-4 are recognized as equivalent to infDCs because of similar morphology, phenotype, and characteristics (75, 76). CCR2 controls the exit of monocytes from the BM and the migration to the site of inflammation and critical for infDCs. Further, MyD88 and TLRs are known to be required for the maturation and migration of infDCs (77, 78).

HUMAN BDCA1 (CD1c)$^+$ DCs

Dendritic cells isolated from human are identified as Lin$^-$ (CD3, CD19, CD14, CD20, CD15, glycophorin A) CD11c$^+$HLADR$^+$ cells (79) and are classified into three groups based on their expression of BDCA1, BDCA3, and CD16 (60). BDCA1$^+$ (CD1c$^+$) DCs are one of the blood DC subsets found, in addition to lymphoid tissue-resident DCs and those observed in the skin of humans (79–81). BACA-1$^+$DCs are likely the human counterpart of murine CD11b$^+$DCs (82). BDCA1$^+$DCs have a strong capacity for allostimulation (79) and can cross-present exogenous antigen to CD8$^+$ T cells but less efficiently than BDCA3$^+$DCs (46–48, 83–85).

DC CHIMERISM AFTER HUMAN ALLOGENEIC HCT

Although the replenishment of recipient DCs depends on donor hematopoietic stem cells (HSCs) and associated precursors, the exact half-life of host APCs in especially inflamed tissues is not well-understood. So far, kinetics of DC engraftment and turnover (DC chimerism) utilizing myeloid specific or directly staining DCs in peripheral blood mononuclear cells (PBMCs) after allo-HCT, have been reported in humans (86–99). Most of these reports demonstrated that the reconstitution of human DCs (myeloid CD11c$^+$DCs and plasmacytoid CD123$^+$DCs) in the early phase of allo-HCT show that nearly complete donor-derived chimerism (CDC) develops and maintains in the late phase. However, a small population of recipient-derived DCs may exist long-term (86, 90, 93, 94). Interestingly, patients with acute GVHD showed significantly lower donor chimerism of DCs as well as low numbers of circulating DCs (93, 94, 96). Further, 6-sulfo lac NAc DCs (slan DCs), potent producers of inflammatory cytokines following LPS stimulation (100) are a major subpopulation of human blood DCs and are also reduced in the patients with severe acute GVHD (92). Although it is helpful to examine the kinetics and chimerism of the peripheral circulating DCs, the kinetics and activation of tissue-resident DC subsets in recipient (especially GVHD-associated organs and/or lymph nodes) might play a role in the development of GVHD.

Host-derived Langerhans cells (LCs) are rapidly depleted by myeloablative regimens and are quickly replaced by donor type in the absence of GVHD. The recovery of donor LC chimerism and numbers, however, are delayed in the presence of acute GVHD (98, 99). In the skin, host-derived myeloid DCs (such as CD1a$^+$ and CD14$^+$DCs) are quickly replaced by donor cells, where host-derived macrophages still exist during GVHD (97). Similar to the relationship between GVHD and DC kinetics, a decrease in number of DCs is observed (96) and mixed chimerism in DCs has the

capacity for a potent GVT effect in donor lymphocyte infusion (DLI) (101). This suggests a positive impact of host-derived DCs on GVT effect.

DCs AFTER EXPERIMENTAL ALLOGENEIC HCT

The results from experimental allo-HCT suggest a complicate role for DCs in GVHD. For instance, cDCs and pDCs are activated by TBI (102) and inflammatory cytokines (103, 104) (IL-1 and TNF-α), which are released by damaged tissues. These activation signals up-regulate the expression of antigen presenting and co-stimulatory molecules and could modulate GVHD (102). Moreover, when all other hematopoietic APCs are absent, DCs alone may induce GVHD (5, 105). However, recent reports indicate that host-derived hematopoietic APCs are dispensable for inducing GVHD, specifically $CD11c^+$DCs and/or pDCs depletion in the presence of other APCs (106, 107) does not attenuate GVHD, it might even increase lethal GVHD (15, 107). These data clearly demonstrate that host DCs are therefore not crucial for the induction of GVHD and could even play a regulatory role. On the other hand, donor-derived APCs, especially cDCs too are not required for induction of GVHD, but may play a role in maintenance or aggravation of GVHD in presence of other hematopoietic APCs (6, 106).

DCs AND GVT

To maximize GVT responses, two important factors must be considered: antigen presentation and donor T cells. Although both host and donor APCs have been shown to play an important role in GVHD, their role in GVT is only beginning to be understood. Donor T cells have to attack recipient tumor cells in GVT. To that end, they must recognize both alloantigens and tumor specific antigens (TSAs) that presented either directly by the tumor or indirectly by the professional APCs (**Figure 1**). There is a large amount of evidence that tumor themselves are generally poor presenters and activators of T cell effector responses. In the context of allo-HCT, professional APCs are required for GVT. Their requirement, however, when certain leukemia or tumors may efficiently present antigens to donor T cells have not been obviously analyzed. Nonetheless, GVT responses are optimal when both alloantigens and TSAs responses are induced (7). While alloantigen responses are also elicited by many APCs including both hematopoietic-derived and non-hematopoietic-derived APCs cause GVHD, TSAs are exclusively directed to tumors and thus considered to GVT without concomitantly causing GVHD. In cases where tumors are poor APCs of TSAs to donor T cells, the TSAs likely have to be efficiently taken up and cross-presented on professional APCs. In this regard, DCs may be most relevant and could employ three possible mechanisms they possess better than other hematopoietic APCs, capability for better cross-presentation and cross-dressing.

Clinically, most patients with allo-HCT receive HSCs and T cells from human leukocyte antigen (HLA) matched, but multiple minor histocompatibility antigens (MiHAs) mismatched donors. This difference in MiHAs between host and donor are targets for donor T cells to mediate GVH responses. Alloantigen is expressed by all host APC subsets as endogenous antigen, which they directly present to donor $CD8^+$T cells, even if the interaction is brief (4). In addition to MiHAs, donor T cells respond to TSAs that are virally encoded and/or mutated tumor antigens representing additional important targets for GVT responses. Activated and proliferated allogeneic T cells, stimulated by APCs, are "double edged swords" in that they not only attack host residual tumors but also damage normal host tissues. Augmenting GVT responses through identification of relevant TSAs and determining T cells that specifically respond to them is clinically challenging because GVHD is an allo-reactive disease enhancing TSA-specific T cell responses, which are dependent on allogeneic reactions (108, 109). As one approach to distinguish this clinical dilemma, recently, MHC class I-associated tumor-specific phosphopeptides presented on hematological tumors were shown to be critical for induction of their specific memory-like $CD8^+$T cells against leukemia and that the response against leukemic patients can be restored after allo-HCT (110). These suggest that DCs must simultaneously present both alloantigens, derived primarily from the endogenously polymorphic peptides in the host target tissues, and TSA to donor $CD8^+$ and $CD4^+$ T cells via MHC class I and class II molecules, respectively. In the clinic, the importance of host APCs in GVL has been suggested in patients with mixed chimerism after DLI in non-myeloablative BMT (111).

We and others have experimentally explored the role of APCs in GVL. Host type APCs are required to maximize GVT responses after allo-HCT (7) and after DLI because they prime donor CTL in an effective manner (112–115). Host MHC class II^+ APCs and $CD4^+$ T cells have an indispensable role in CTL responses in mixed chimera models (112). In addition, donor T cells primed by leukemia lysate-pulsed host APCs before DLI, enhance GVT responses in either leukemia-bearing full chimera or mixed chimera models (113). These data suggest that the host environment is critical for mediating GVT responses. Host type sialoadhesin$^+$ macrophages, which increase inducible nitric oxide (iNOS) production by CD40–40L interaction in the liver, stimulate CTL and prevent liver metastasis (116, 117). Based on the fact that host leukemia cells or tumors express alloantigens, in addition to TSA, may possess co-stimulatory molecules, they could be "APCs." Although they express APC like features, they have likely undergone a process of "immune-modulating," making them poor direct stimulators of an effective T cell response using a variety of immune-suppressive mechanisms. We have shown that certain lymphoma cells lines, despite some APC features, are not capable of driving an efficient GVT response in the absence of hematopoietic-derived APCs (7).

We have explored, more recently, the APC subsets that are required for optimal GVT without GVHD. We recently found that host-derived $CD8\alpha^+$DCs are required for the induction of optimal GVT responses utilizing Batf3 deficient mice as recipients in experimental allo-HCT (8). We also found that TLR3 stimulation via poly I:C in host $CD8\alpha^+$DCs, enhanced GVL responses without exacerbating GVHD (8). As we described previously, $CD8\alpha^+$DCs are critical for cross-presentation of tumor and viral antigens (32, 118, 119) because of their well-specialized cross-presentation capacity and their superior ability to prime antitumor CTL responses (32, 119–121) without enhancing GVHD (8, 122). As noted above, recently human $BDCA3^+$, $XCR\text{-}1^+$, $DNGR\text{-}1^+$DCs found in spleen, blood, and non-lymphoid tissues are recognized as the equivalent of murine $CD8\alpha^+$DCs by multiple

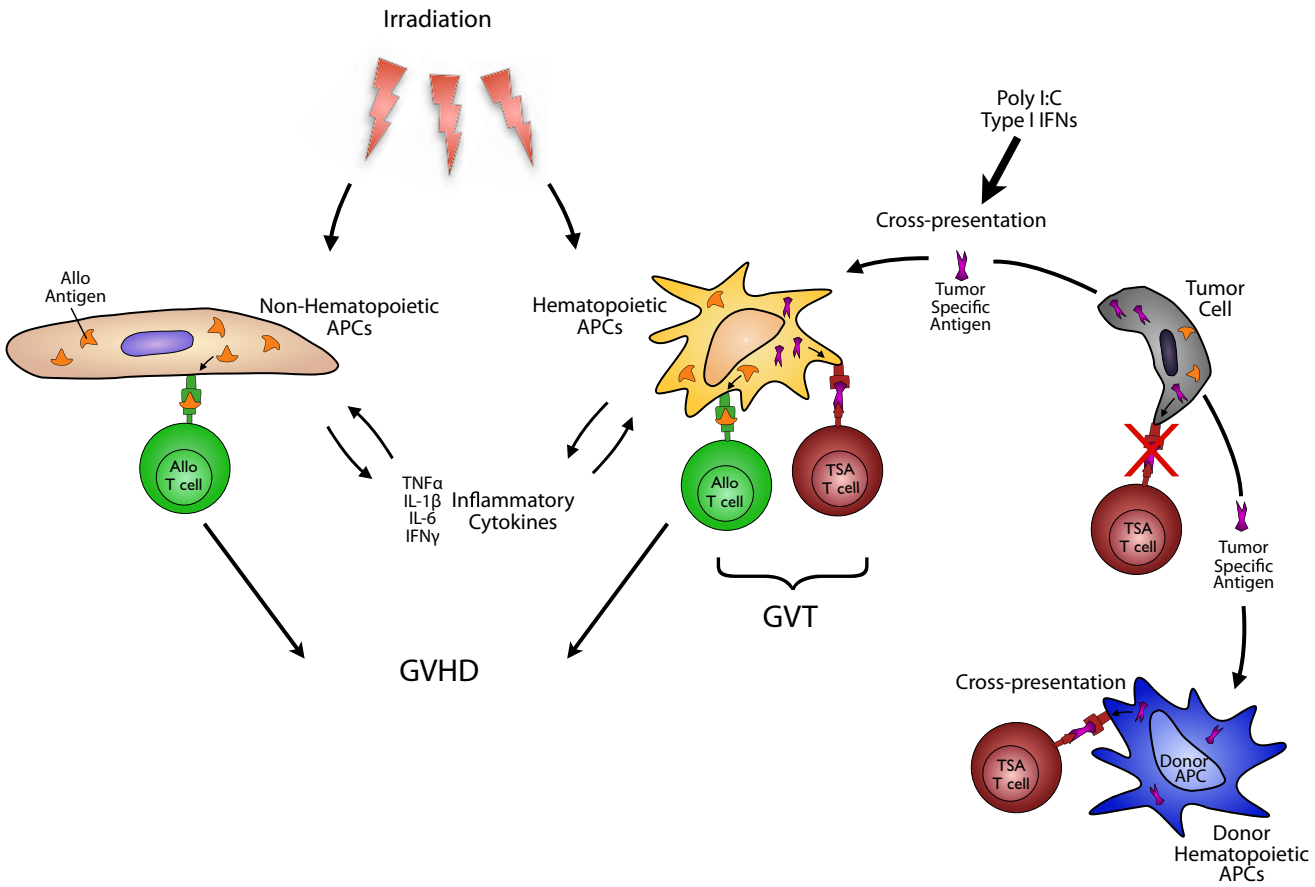

FIGURE 1 | Antigen presenting cells in GVT: to maximize GVT responses, two important factors must be considered: antigen presentation and donor T cells. Although both host and donor DCs have been shown to play an important role in GVHD, their role in GVT is only beginning to be understood. To induce GVT, although donor T cells must recognize both alloantigens and TSAs that presented either directly by the tumor or indirectly by the professional APCs, tumor themselves are generally poor presenters and activators of T cell effector responses. Therefore, professional APCs are required for optimizing GVT. While alloantigen responses are also elicited by many APCs including both hematopoietic-derived and non-hematopoietic-derived APCs cause GVHD, TSAs are exclusively directed to tumors and thus considered to GVHD without concomitantly causing GVHD. In cases where tumors are poor APCs of TSAs to donor T cells, the TSAs have to be efficiently presented by professional APCs, especially DCs, derived from either donor or host. This mechanism of presentation includes cross-presentation.

investigators (26, 46–48, 123). Therefore, our investigations underscore the principle of enhancing antigen presentation using a subset of host APCs as a strategy for effective enhancement of GVT responses following allo-HCT. However, cellular processes of regulating GVT responses in host APCs still remain unclear. Specifically whether low numbers of CD8α$^+$DCs reduce TSA responses or decrease GVT responses remain unknown. We also explored the molecular mechanism in hematopoietic-derived APCs for enhancing GVHD. The absence of Ikaros in host hematopoietic APCs exacerbates GVHD, but without concomitantly enhancing GVT responses in multiple models (unpublished data). This uncoupling is an interesting phenomenon as GVT responses are usually tightly linked with GVHD severity. Furthermore, genetic alteration of Ikaros family zinc finger protein 1 (IKZF1) in acute lymphoblastic leukemia (ALL) is associated with poor outcome and high relapse after chemotherapy (124, 125). Therefore, we are pursuing whether Ikaros in leukemic cells alone or both leukemic and non-leukemic host hematopoietic cells play a role in mediating GVT resistance.

Understanding the host microenvironment, especially that of the tumor is essential for GVT studies. Tumor-infiltrating DCs in tumor microenvironments in hosts are suggested to regulate CTL responses, however, their role in the context of allogeneic HCT remain obscure.

The role of donor-derived DCs in mediating GVT is also being explored. Initial reports regarding this association demonstrated that donor APCs are not required for GVT responses, but play an indispensable role in GVHD in MHC matched, MiHA mismatched BMT model (6). In order to present host TSAs via donor APCs to donor CD8$^+$T cells, donor APCs must have the capacity for cross-presentation as they do not express both endogenous alloantigens and TSAs. Furthermore, additional studies are needed to determine which specific subsets of donor APCs play a critical role in enhancing GVT responses. Reports suggest that donor CD11b$^-$ APCs within the BM grafts consist mostly of pDC progenitors (pre-pDCs) and enhance GVT activity of donor T cells by promoting differentiation into Th1/type 1 CTLs. These effects have further

been shown to be mediated by IL-12 in murine allo-HCT models (126, 127). Pre-pDCs also regulate GVH and GVT responses altering the balance between donor Tregs and inflammatory T cells by inducing indoleamine 2,3-dioxygenase (IDO) synthesis (128). In humans, however, there are no data of the exact mechanisms of specific subsets of donor APCs in GVT. Therefore, studies examining and elucidating the kinetics of these subsets of DCs would contribute to likely better understanding the mechanism of GVT in humans.

Recent reports suggest a paradoxical association between CMV reactivation after allo-HCT and reduced disease relapse (129–131). The mechanisms that CMV reactivation induces potent GVT are still unclear. However, donor APCs and NK cells might play an important role in this interesting phenomenon (132). Interaction between cDCs and NK cells is critical to the activation of effective antiviral or antitumor response (133, 134). It is possible that donor DC–NK cell interactions might play a role in enhancing GVT mediated by NK cells in this context.

CROSS-PRESENTATION AND GVT

Dendritic cells are well-known to take up exogenous antigens via endocytosis or phagocytosis. Antigen is then processed in the endoplasmic reticulum (ER) and presented via Class I molecules. These processes are known as cross-presentation. Although the molecular mechanism of cross-presentation is still under investigation, two major intracellular pathways of cross-presentation are speculated. One is cytosolic and the other one is a vacuolar pathway (135). The cytosolic pathway depends on the proteasome, which degrades internalized proteins in the cytosol. The degraded peptides are then transported into the ER in a transporter associated with antigen processing 1 (TAP1) and TAP2-dependent manner. Peptide is then either loaded onto MHC class I molecules (ER loading) or re-imported into the phagosome to be loaded onto MHC class I molecules (phagosomal reloading) (135). A novel molecular mechanism utilizing the small GTPases Rac1 ($CD8\alpha^-$DCs) and Rac 2 ($CD8\alpha^+$DCs), regulate phagosomal oxidation, which is critical for the cross-presentation capacity (136). In addition, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) Sec22b plays an important role in phagosomal function through the recruitment of ER proteins into the phagosome (137) and heat shock protein 90 (HSP90) contributes to cytosolic translocation of extracellular antigen, enhancing cross-presentation (138). Conversely, the vacuolar pathway is known to be a TAP and proteasome independent pathway (139–141) where exogenous antigens are degraded in the phagosome and subsequently loaded on MHC class I. This pathway is sensitive to cathepsin S inhibitors (140). Some DCs, such as those that express $CD8\alpha^+$ (32, 37, 142, 143), $CD103^+$ (144–147) in mice, and $BDCA3^+$DCs (functional homology to mice $CD8\alpha^+$DCs) (46, 123, 148–150) in human are known to have the capacity for cross-presentation. However, some recent reports suggested that nearly all DCs have the capacity for cross-presentation depending on the source of antigen, cytokine milieu, and expression of immunoreceptors specialized to take up exogenous antigens (76, 83, 149, 151). The role of cross-presentation in GVT responses is still unknown. Our data indicates a role for $CD8\alpha^+$DCs and also suggested that TLR3 agonist, polyI:C, can increase GVT without enhancing GVHD in host DC-dependent manner (8). Therefore, we presume that specialized DCs could be associated with optimizing GVT responses because mouse $CD8\alpha^+$DCs and human $BDCA3^+$DCs possess the most potent cross-presentation capacity of TSAs. However, direct *in vivo* demonstration enhancing cross-presentation by $CD8\alpha^+$DCs or TLR3 agonist in increasing GVT has not been shown. While these are being explored, at the minimum our data suggested a novel concept that it is feasible to modulate host DCs to improve GVT without increasing toxicity. It remains to be tested, however, whether this concept holds true for all leukemia or tumors. In any event, it does suggest a window of opportunity for careful design of clinical trials in high-risk leukemia.

CROSS-DRESSING AND GVL

Recently, another means of antigen presentation, called "cross-dressing" was forward by Ostrand-Rosenberg's group in 2006 (152, 153). It is postulated that cross-dressing transfers cellular materials (such as peptide MHC to DCs) triggering DC activation and enhanced tumor-specific $CD4^+$ T cells in cancer vaccine (153). In 2011, as a breakthrough mechanism of elicited CTL responses by DCs, preformed peptide MHC class I complex is expressed on infected cells and can be transferred to uninfected DCs without requiring other antigen processing. This process mediates the activation of memory $CD8^+$ T cells after viral infection (154). $CD8\alpha^+CD103^+$DCs are thought to play an important role in not only cross-presentation but also cross-dressing to prime CTLs following vaccination (155). Its role suggested in GVHD but GVT responses is still unknown.

THE STRATEGY OF AUGMENTING GVT RESPONSES UTILIZING DCs

Graft-versus-tumor is tightly linked with GVHD and is very difficult to uncouple the two. However, recent advances and understanding of DC biology make treatment regimens previously not considered, namely modulating antigen presentation, to now be practical options. Nonetheless much remains to be understood. Specifically, comprehensive understanding of DC subsets will enable us to maximize GVT responses. For instance, either by enhancement of cross-presentation, increased NK cell activation, or induction of type I IFN etc.

We and others have shown that administration of poly I:C stimulates TLR3 on $CD8\alpha^+$DCs enhancing cross-presentation and direct presentation to CTLs against tumors and virus infection (8, 118). In addition, poly I:C administration also activates NK cells through the enhancement of myeloid DC–NK interaction mediated through an IRF-3-toll/interleukin 1 receptor homology domain-containing adaptor molecule (TICAM-1)-IRF-3-dependent NK-activating molecule (INAM) axis-dependent manner (134). Moreover, $CD8\alpha^+$DCs treated by poly I:C can activate NK cells in the IFN-promoter stimulator-1 (IPS-1) and Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF)-dependent manner (156). Therefore, poly I:C treatment after allo-HCT could be extended to increase GVT, however, poly I:C in this context must be carefully studied as it may enhance GVHD.

Careful utilization of exogenous type I IFN (IFN-α/β) administration may also be a valuable method of enhancing GVT responses because they play an important role

in cross-presentation of tumor antigens on DCs, especially CD8α$^+$DCs, and enhance CTL responses (119, 120, 157). In murine allo-HCT models, exogenous type I IFN administration augments CTL responses through the increased sensitivity of host target tissues and leukemia to respond to cell mediated cytotoxicity in CD8-dependent GVHD/GVT model regardless of decreasing GVHD response in CD4-dependent model (158).

Other strategies to enhance antitumor responses through the augmentation of the cross-presentation capacity of TSA and activation of CTLs may also be feasible. Alpha-alumina nanoparticles (159), poly (γ-glutamic acid)-based nanoparticles (γ-PGA NPs) (160), Fcgamma-receptor (FcγR) antigen targeting (161), TLR7 stimulation by polyuridylic acid (polyU), which is a synthetic ssRNA analog (162), vitamin E analog-α-tocopheryl oxyacetic acid (α-TEA) (163) may be useful, but have not been studied in GVT models. Modulation of host type DCs with anti-CD3 pre-conditioning is also an efficient strategy for separating GVT and GVHD (164). Furthermore, recent modulation of DCs by reagent-based inducible or constitutive methods suggested that deep deletion of host cDCs, pDCs, and B cells are dispensable for decreased GVH responses (107). This indicated that very low numbers of DCs, or all host cells including non-hematopoietic APCs, can directly present alloantigen. Alloantigen expression on host non-hematopoietic cells decreases GVT responses in a PD-1/PD-L1-dependent manner in murine experimental BMT (165). Given this, enhancement of function in only certain DCs specialized for TSA presentation may also increase GVT responses without exacerbating GVHD. Moreover, experimental data suggested that modulation of DC function with HDAC inhibitor can result in immunomodulation to reduce GVHD (166).

Aside from enhancement of the presentation capacity in DCs, disruption of negative regulatory interactions is also important for GVT responses. PD-1/PD-L1 interactions and CD47–SIRP-α interactions are thought to be critical immunosuppressive function in the tumor environment. For instance, because the expression of PD-1 on T cells and PD-L1 on APCs facilitated increased Tregs and decreased CTL functions, PD-1/PD-L1 blockade with anti-PD-L1 monoclonal antibody decreased the infiltrating number of Tregs and increased the number and function of tumor reacting CTLs in an AML mouse model (167). Furthermore, knock down of PD-L1 and PD-L2 on MoDCs by utilizing siRNA demonstrated augmented expansion and function of MiHA-specific memory and effector CD8$^+$ T cells from leukemia patients *in vitro* (168). These data suggested that anti-PD-L1 and PD-L2 blockade might be a potential strategy for the enhancement of GVT responses. Tumors may also escape from tumor surveillance utilizing the interaction between monocytic CD47 and SIRP-α, which is an inhibitory receptor of phagocytosis (169). Recent report showed engineered high affinity SIRP-α variants can disrupt this interaction and increase phagocytosis of cancer cells and enhance antitumor response (170). Although we do not know how these pathways affect GVHD, such strategy may also be considered as potential option to treat patients with high risk leukemias.

CLOSING REMARKS

Dendritic cells play important roles in both GVHD and GVT. Because DCs are heterogeneous, the role of specific DCs in GVHD and GVT in the presence or absence of other hematopoietic-derived APCs will need further examination. Identification of a specialized subtype of DC that may increase GVT without enhancing GVHD, such as CD8α$^+$DCs in mice, may be possible. Functional studies have identified direct antigen presentation capacity, cross-presentation, and cross-priming of CTLs as critical mechanisms in allo-HCT. To enhance GVT response, both alloantigen and TSA must be presented to CTLs. However, tumor cells themselves have a poor antigen presentation capacity, therefore TSA are cross-presented by APCs. Enhancement of the cross-presentation capacity has the potential to increase GVT response and be a presumably new strategy in allo-HCT. Through the utilization of DCs, the goal of increasing GVT and diminishing GVHD might be realized.

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Antigen-specific T-cell activation independently of the MHC: chimeric antigen receptor-redirected T cells

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Adoptive T-cell therapy has recently shown promise in initiating a lasting anti-tumor response with spectacular therapeutic success in some cases. Specific T-cell therapy, however, is limited since a number of cancer cells are not recognized by T cells due to various mechanisms including the limited availability of tumor-specific T cells and deficiencies in antigen processing or major histocompatibility complex (MHC) expression of cancer cells. To make adoptive cell therapy applicable for the broad variety of cancer entities, patient's T cells are engineered *ex vivo* with pre-defined specificity by a recombinant chimeric antigen receptor (CAR) which consists in the extracellular part of an antibody-derived domain for binding with a "tumor-associated antigen" and in the intracellular part of a T-cell receptor (TCR)-derived signaling moiety for T-cell activation. The specificity of CAR-mediated T-cell recognition is defined by the antibody domain, is independent of MHC presentation and can be extended to any target for which an antibody is available. We discuss the advantages and limitations of MHC-independent T-cell targeting by an engineered CAR in comparison to TCR modified T cells and the impact of the CAR activation threshold on redirected T-cell activation. Finally we review most significant progress recently made in early stage clinical trials to treat cancer.

Keywords: chimeric antigen receptor, T-cell receptor, adoptive cell therapy, antibody, antigen-presenting cell

TUMOR-SPECIFIC T CELLS FOR ADOPTIVE CELL THERAPY

Experimental and clinical evidences indicate that the immune system is capable of identifying and destroying cancer cells in a specific fashion; tumor-infiltrating lymphocytes (TILs), expanded *ex vivo* and re-administered to the patient, exhibit a powerful anti-tumor response and induce an acute inflammatory reaction which attracts a second, antigen-independent wave of immune cell invasion into the same lesion. Adoptive TIL therapy has shown some success in the treatment of chemotherapy resistant melanoma, even in advanced stages of the disease (1). The procedure, however, is technically challenging since it involves the isolation of T cells from melanoma biopsies and their amplification *ex vivo* to therapeutic numbers; not every melanoma biopsy provides TILs and allows sufficient expansion. Moreover, the range of TIL bearing malignant lesions, apart from melanoma, is small limiting the application of the strategy to a broad variety of cancer entities.

The implementation of redirected T cells in cancer therapy is based on engineering T cells with pre-defined specificity to target virtually every cancer cell and on the production of engineered T cells in therapeutic numbers. To provide specificity peripheral blood T lymphocytes were *ex vivo* engineered with a recombinant T-cell receptor (TCR) of known specificity which recognizes cognate peptide-loaded major histocompatibility complexes (pMHC) of a so-called tumor-associated antigen (TAA). Such TCR engineered T cells showed promise in clinical trials (1, 2). Some conceptual deficits, however, limit the broad application of TCR engineered T cells including the HLA restriction, the dependency on adequate major histocompatibility complex (MHC) expression by tumor cells, the limited number of peptide-MHC complexes identified so far which can be used for screening and the potential mispairing with the endogenous TCR producing novel, unforeseen specificities which might induce severe auto-immunity after adoptive transfer (3).

Whereas the T-cell therapy using *ex vivo* expanded patients' TILs leads to significant clinical effect in patients with metastatic melanoma (1), difficulties are arising when engineering T cells with a recombinant TCR, in particular when non-immunogenic tumor-associated self-antigens are targeted (4). In a pre-clinical tumor model the treatment with TCR engineered T cells alone was without effect while the combination of vaccination with TCR modified T-cell transfer was synergistic.

In this situation, Zelig Eshhar, Weizmann Institute, proposed to redirect T cells by a recombinant receptor molecule, a chimeric antigen receptor (CAR), which in the extracellular part consists of an antibody with pre-defined binding specificity to a broad variety of targets and in the intracellular part of a T-cell activation domain (5). Such CAR modified T cells became generally known as "T-bodies" (5). In contrast to the TCR, the archetypical CAR is composed of one polypeptide chain (**Figure 1**). The binding domain is mostly a recombinant antibody in the single chain format consisting of the variable domain of the heavy and light chain linked by a short synthetic peptide (scFv). The extracellular part of a receptor molecule, for instance the NK cell-derived NKG2D ligands (6) and the surface NKp-30 (7) receptor, were also successfully integrated into the conventional CAR structure instead of the classical antibody-derived binding domain. The CAR intracellular

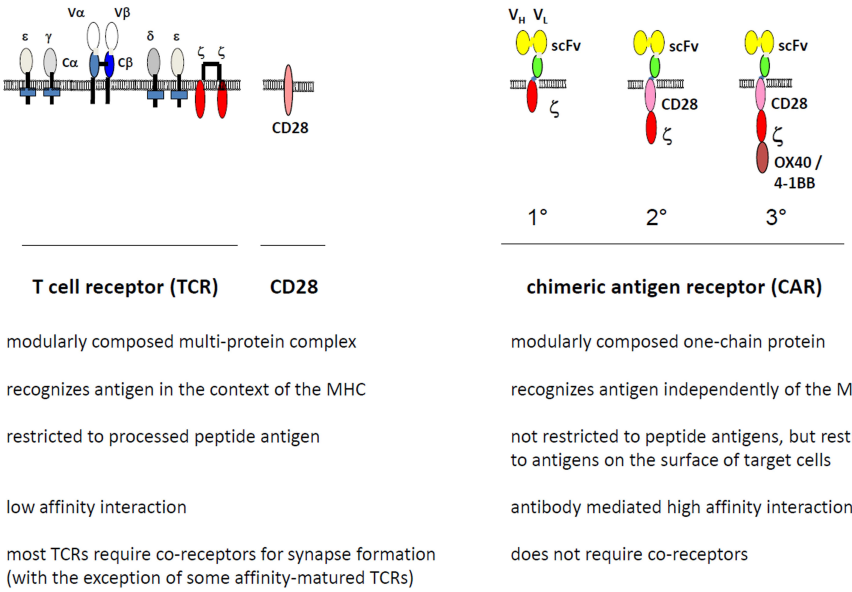

FIGURE 1 | Modular composition of the chimeric antigen receptor (CAR) compared to the T-cell receptor (TCR). The TCR binds to cognate peptide-loaded MHC (pMHC) by the TCR α and β chains, forms the immunological synapse by clustering accessory components including CD3ζ and CD28, and initiates the downstream signaling pathway for T-cell activation through phosphorylation of the CD3ζ ITAM motives. The CAR, in contrast, is composed of one polypeptide chain; the extracellular single chain fragment of variable region (scFv) antibody domain binds to the target antigen in a MHC-independent fashion. Upon CAR clustering, the intracellular CD3ζ chain, with or without costimulation through members of the CD28 family, initiates the downstream signaling for T-cell activation. Co-receptors may modulate CAR activity. In contrast to a first generation (1°) CAR, second (2°), and third (3°) generation CARs harbor in addition one or more costimulatory moieties in their intracellular part.

signaling domain is preferentially derived from the CD3 ζ-chain of the TCR/CD3 complex or, alternatively, from the γ-chain of the high affinity IgE Fc receptor-I (FcεRI). Binding with cognate antigen on the tumor cell surface results in CAR clustering on the engineered T-cell with the consequence that the immunoreceptor tyrosine-based activation motifs (ITAMs) of the signaling moiety become phosphorylated and initiate a downstream signaling cascade which finally induces T-cell amplification, cytokine secretion, and cytolytic activity of the CAR T-cell toward the cognate tumor cell.

CAR T cells overcome some limitations of the TCR-based strategy by targeting cells in an MHC- and dendritic cell (DC)-independent fashion. The properties of a CAR and TCR differ substantially in respect to target binding and subsequent T-cell activation. For instance these are in particular the TCR avidity for a given pMHC, the number of MHC molecules, the availability of co-receptors and the moderate TCR affinity for the cognate MHC peptide complex compared to the high affinity of the antibody in a CAR. On the one hand the use of antibody-based CAR T cells enables the targeting of antigens of different composition and structure such as peptides, carbohydrates, or inorganic compounds, and on the other hand, the TCR recognizes peptide antigens exclusively in the context of the particular MHC and thereby faces a limited variability. TCRs are moreover inherently cross-reactive toward endogenous antigens (8). The potential CAR targets thereby far outnumber their MHC presented counterparts which can be recognized by TCR modified T cells. In this report we review some advantages and limitations of MHC-independent target recognition by CAR T cells and review most significant progress recently made in early stage clinical trials to treat cancer.

THE CAR STRATEGY: ANTIBODY-MEDIATED, MHC-INDEPENDENT ANTIGEN RECOGNITION BY ENGINEERED T CELLS

The design of the antibody-derived CAR differs in several major features from the TCR which physiologically mediates target recognition by T cells (**Figure 1**). By using an antibody for binding, T cells gain antibody-defined specificity: (i) T cells without CAR or equipped with a CAR of different specificity are not activated by the target cells; (ii) the cognate antigen needs to be on the cell surface to trigger CAR T-cell activation, intracellular antigens are not recognized by the CAR; and (iii) CAR-mediated T-cell activation can be specifically blocked by an antibody directed toward the CAR binding domain (9).

As a consequence of using an antibody for binding, CARs can redirect T cells toward targets of any chemical composition or conformation as far as an antibody is available. Indeed, CARs were engineered which target T cells toward carbohydrate antigens like CA19-9 (10–12). The TCR, in contrast, is restricted to the recognition of specific peptides presented by the particular MHC. Antibody-mediated target recognition by CARs, however, does not exclude targeting MHC presented antigens. Using an antibody which recognizes NY-Eso-1 peptide (157–165) in the context of HLA-A*0201, Stewart-Jones et al. engineered a CAR recognizing the MHC presented peptide analog SLLMWITQV (13). The antibody domain used for CAR targeting was optimized by

modification of the individual amino acids which interact between the antibody and the peptide providing an antibody with 20-fold improved affinity, exceeding the affinity of the respective TCR by about 1000-fold. The high affinity antibody when engineered as recombinant CAR on T cells conferred specific killing of HLA-A*0201/NY-ESO-$1_{157–165}$ target cells as do T cells modified with the corresponding TCR.

BOTH CD8$^+$ AND CD4$^+$ T CELLS CAN BE REDIRECTED IN A MHC-INDEPENDENT FASHION

By bypassing MHC class I and class II restriction by an antibody-derived binding domain, CAR engineered T cells of both CD8$^+$ and CD4$^+$ subsets can be recruited for redirected target cell recognition (9, 14, 15). Equipped with a CAR, CD4$^+$ T cells showed as cytolytic as do CD8$^+$ T cells toward CAR-defined target cells. While human CD8$^+$ T cells predominantly use two pathways in executing cytolysis, i.e., perforin and granzyme exocytosis and to some extend death receptor signaling via Fas/Fas-ligand (Fas-L) or TNF/TNF-receptor (TNF-R) (16), the mechanism of CAR-mediated lysis by redirected CD4$^+$ T cells was a matter of debate for some time. Investigations utilizing mutant and knock-out mice suggest that MHC class II restricted cytolysis by murine CD4$^+$ T cells is predominantly mediated by the death receptor system (17, 18) which is in contrast to MHC class I restricted cytolysis by CD8$^+$ CTLs relying mainly on perforin and granzymes. Accordingly, murine CD8$^+$ T cells engineered with a CAR lyse Fas resistant target cells whereas CD4$^+$ T lymphocytes do not (19). In contrast to murine cells, CAR redirected human T cells mediate cytolysis predominantly by granzyme/perforin which can be executed independently of Fas or TNF-α signaling (14). The extent in redirected cytolysis correlates with the amount of cytolytic effector molecules; CAR CD4$^+$ T cells which harbor about half amounts of perforin and granzyme B required about twice the number of effector cells to achieve the same cytolytic efficacy compared to CAR redirected CD8$^+$ T cells. CAR CD4$^+$ T cells rapidly lyse their targets in a short term *in vitro* cytotoxicity assay as do engineered CD8$^+$ T cells which is in accordance to a perforin mediated process whereas death receptor signaling induces cytolysis of the delayed type. CAR engineered CD4$^+$ T cells lyse both Fas- and TNF-resistant target cells. The observation is in accordance to a report that non-modified human CD4$^+$ T cells execute cytolysis predominantly by granule exocytosis and not by the Fas/Fas-L system (20). In contrast to CAR modified cells, CD4$^+$ cells engineered with a MHC class I restricted TCR were reported to lyse exclusively those target cells that are susceptible for death receptor signaling (21). Both studies, however, differ in several issues including the use of a MHC class I-dependent TCR vs. a MHC-independent CAR for redirecting T cells. As a consequence for adoptive cell therapy, CAR engineered patient's CD4$^+$ T cells can efficiently provide help upon CAR-mediated activation and can eliminate tumor cells in a direct fashion and independently from MHC class II restriction.

"AFFINITY CEILING" OF ANTIBODY-MEDIATED CAR T-CELL ACTIVATION

CAR-mediated T-cell activation is thought to depend on and to increase with the binding affinity to cognate antigen; however, the interaction is likely of higher affinity than binding of the physiological TCR to peptide loaded MHC. Two studies addressed in detail the situation (22, 23). The Chmielewski study (22) made use of a panel of CARs of the same backbone and same epitope specificity but with different binding affinities. The affinities were in the broad range of 10^{-7}–10^{-11} M and were obtained upon mutation of the parental antibody while preserving the binding specificity. CAR T-cell activation correlated with the affinity of the antibody binding domain when the target antigen is present in an immobilized fashion coated onto surfaces. In contrast, when the cognate antigen is present on the surface of the target cell, the CAR-mediated cytotoxic effect on target cells and the release of IFN-γ and IL-2 did not increase with the binding affinity above threshold, which was in that example about $K_D = 10^{-8}$ M. While the conditions that define the activation threshold, however, are so far not understood on the molecular level, the study makes clear that furthermore increase in affinity above threshold does not improve the redirected T-cell attack toward target cells but may result in antigen-independent T-cell activation.

The Hudecek study (23) evaluated scFv's of different affinities and CARs with different backbones with respect to their efficacy in redirecting T cells. The CAR with higher binding affinity conferred maximum T-cell activation with respect to cytokine release and proliferation compared to the CAR with lower affinity. The redirected cytolytic activity, however, was nearly the same. Although the study confirms previous observations that increase in affinity does not necessarily improve all T-cell effector functions, the comparison of the CARs is alleviated in that two binding domains targeting different epitopes, although in the same domain of the targeted ROR1 molecule, were used.

A recent study explored the situation for TCR modified T cells to determine the affinity threshold with respect to the optimal balance between anti-tumor efficacy and auto-immunity (24). Similar as for CAR modified T cells, TCR redirected anti-tumor activity shows a plateau at a defined TCR affinity, likely due to diminished contribution of TCR affinity to avidity above the threshold. Additional differences probably lie in the ability of different affinity interactions eliciting different effector functions at different antigen concentrations. The observations are in accordance to the CAR situation and strongly suggest that a relatively low affinity threshold is mandatory to avoid self-damage, that high affinity TCRs do not necessarily improve efficacy given the close relationship between anti-tumor activity and auto-immunity.

THE POSITION OF THE TARGETED EPITOPE MATTERS: MEMBRANE PROXIMAL VS. DISTAL EPITOPES AS TARGETS FOR CAR ENGINEERED T CELLS

By using an antibody for targeting, CAR engineered T cells can be redirected toward a variety of epitopes of the same antigen as far as the epitope is accessible to the respective antibody. The various epitopes of a given membrane-bound molecule, however, are not equally good targets for efficient T-cell activation. This was shown when membrane distal and proximal epitopes of the same membrane-bound molecule were targeted by CARs. For instance, when targeting carcinoembryonic antigen (CEA) expressed on gastrointestinal carcinoma cells, a higher degree of T-cell activation was obtained when epitopes closer to the cell membrane

were targeted (25). The epitope itself is not the cause of the phenomenon since the isolated, solid phase bound CEA induces T-cell activation independently of the epitope position but dependent of the antibody binding affinity. The distal epitope when expressed in a more membrane proximal position activated CAR T cells with higher efficiency than in the distal position indicating that the position effect of the targeted epitope has, at least in this example, prominent impact on T-cell activation. The accessibility of the epitope for binding additionally impacts the efficiency in CAR-mediated T-cell activation; in the case of CEA targeting, however, the epitope accessibility seems not to be limiting because the distal epitope, which is thought to be more accessible than the more proximal epitope, is superior in binding but less capable in mediating CAR activation. This is in accordance with another report which analyzed the impact of the position of the target epitope on the structural requirements of the CAR (26). Basically the same observation was made when targeting B-cell lymphoma associated CD22 by CAR T cells (27, 28). To explain the observation, a kinetic-segregation model, initially proposed by Davis and van der Merwe (29) and hypothesized also to occur in CAR engineered T cells, is currently favored. The model suggests that targeting membrane distal epitopes increases the size of the CAR-ligand clusters, which in turn permits large phosphatase molecules such as CD45 to enter the synapse and to repress TCR signaling which is less the case when targeting the membrane proximal epitope. The model, however, does not exclude that accessibility and flexibility of the targeted epitope itself may also contribute to some extent.

The best suitable target epitope and binding affinity for optimal CAR T-cell activation remains so far to be empirically evaluated in each case. This is of major relevance given the broad variety of potential targets for a CAR in contrast to the TCR, the specificity of which is restricted to MHC presented peptides.

CD28 COSTIMULATION PROVIDED BY SECOND GENERATION CARs: MAJOR DIFFERENCES TO STIMULATION THROUGH APCs

First generation CARs provide exclusively only one signaling domain such as CD3ζ-, ε-, or FcεRI γ-chain to initiate redirected activation of pre-stimulated T cells upon CAR binding with antigen. To prevent engineered T cells undergoing activation-induced cell death and anergy, CD28 costimulation simultaneously to CD3ζ signaling is required. CD28 is the prototype of a family of costimulatory molecules that is physiologically engaged on T cells by binding to the respective ligands on antigen-presenting cells (APCs). The agonistic CD28 ligands B7.1 (CD80) and B7.2 (CD86), physiologically expressed on APCs, are missing on most cancer cells with the consequence that the CD3ζ CAR upon binding to cancer cells does not provide the costimulation required for full activation. The limitation was overcome by linking the intracellular signaling domain of CD28 to CD3ζ in one polypeptide chain of the same CAR (30–33). In this so-called "second generation" CAR the artificial fusion of the CD28 and CD3ζ signaling domains facilitates Lck-mediated CD28 phosphorylation that binds and activates phosphatidylinositol 3-kinase for downstream signaling, resulting in full T-cell activation and IL-2 release. Other costimulatory molecules of the TNF-receptor family including 4-1BB (CD137) and OX40 (CD134) can also be integrated into the same CD3ζ CAR molecule or combined with CD28 in a "third generation" CAR. This type of CAR has the advantage that T-cell costimulation occurs in an APC-independent fashion and is accompanied by suppressing inhibitory and/or strengthening stimulatory signals, each costimulatory signal modulating the T-cell effector function in a specific fashion (34). CD28 costimulation is integrated into most currently used CARs because CD28 sustains survival and prolongs polyclonal expansion of engineered T cells without the need of B7–CD28 engagement (35). CD28 co-signaling induces IL-2 that is used in an autocrine fashion by redirected T cells to increase their amplification (36). CD28-CD3ζ CAR signaling moreover counteracts transforming growth factor-β1 (TGF-β1)-mediated repression in T-cell amplification (37). Both prevention from AICD and increased amplification produce prolonged T-cell persistence and an improved anti-tumor attack. Other beneficial properties and some draw backs were recently discussed in more detail (38). Taking advantage of CD28 of other costimulatory moieties like 4-1BB, second generation CARs are currently being explored in early phase clinical trials.

The impact of CAR provided CD28 costimulation on the threshold of antigen-dependent, APC-independent T-cell activation was addressed by using a panel of CARs targeting T cells in the absence of agonistic CD28 ligands (39). CAR provided CD28 costimulation increases cytokine secretion but does not impact the activation threshold or "affinity ceiling," above which an increase in affinity does not increase T-cell activation. CD28 did not increase sensitivity toward target cells with intermediate or low densities of the respective target antigen. Additional CD28–B7 engagement did not further alter CD28-CD3ζ CAR-mediated T-cell activation. In the presence of a CD3ζ CAR, however, B7 engagement increased IFN-γ secretion indicating that the physiological CD28 costimulation through APCs cooperates with CAR-driven T-cell activation.

Another aspect concerns the fact that most target antigens for adoptive immunotherapy are not exclusively expressed on tumor cells but broadly present on a variety of healthy tissues, although frequently at lower levels. Since CAR provided CD28 costimulation in the absence of APCs does not alter the activation threshold, costimulation does not impact the selectivity of a redirected T-cell attack in peripheral tissues, does not lower the affinity ceiling and antigen-dependent threshold of CAR redirected T cells and thereby protects healthy cells with physiological levels of antigen from a T-cell attack.

With respect to costimulation, there are some fundamental differences in the physiologic vs. CAR-mediated T-cell activation. To induce full T-cell activation, the peptide loaded MHC has to interact with the TCR in a form that allows the appropriate synapse formation on the T-cell and to recruit costimulatory molecules which increase stability during early stages in this process. CD28 recruitment by B7 engagement on APCs sustains formation of the immunological synapse which is accompanied by lower amounts of antigen required for T-cell activation. During T-cell–APC interactions, in particular during early activation events, CD28–B7 binding potentiates synapse formation by increasing the density of the synapse components through approximation of the interacting membranes (40); increased clustering integrates the TCR with costimulatory signaling which can compensate for weak TCR

signals (41). The TCR binding threshold exhibits a sharp cutoff between full T-cell activity and no activity; the activation efficiency correlates with the TCR binding to the cognate peptide–MHC on APCs (42). Optimal CD28 costimulation occurs upon high-avidity engagement of dimeric B7.1, followed by dimer dissociation, CD28 down-regulation, and B7.1 internalization (43). CD28-B7 interactions with APCs sustain synapse formation which facilitates T-cell signaling upon low affinity target engagement depending on the extend of supra-molecular clustering (44). This mechanism is in contrast to CD28 CAR-mediated T-cell activation, in particular, the avidity of CAR binding is generally higher than of physiological TCR–MHC interactions. Whether the CAR synapse is formed in the same way as the TCR recruits additional components is so far not resolved. There are, however, some cooperative interactions between the CAR and downstream signaling molecules since additional B7.1-CD28 costimulation improves cytokine secretion initiated by CAR signaling.

CAR BASED ADOPTIVE CELL THERAPY GAINED SUBSTANTIAL SUCCESS IN RECENT EARLY PHASE TRIALS

Adoptive cell therapy with CAR engineered T cells is currently being evaluated in a number of early phase trials, some of them are listed in **Table 1**. Patient's T cells are modified *ex vivo* by retro- or lenti-viral gene transfer with the respective CAR, amplified to therapeutically relevant numbers and given back to the patient by transfusion. Some of these trials produced encouraging evidence of clinical efficacy. CD19-specific CAR T cells induced complete and lasting remission of refractory $CD19^+$ B-cell chronic lymphocytic leukemia (CLL) in all of the first three reported patients (44, 45). When successfully engrafted, CAR T cells expanded *in vivo* more than 1000-fold compared to the initial level, persisted in the peripheral blood and bone marrow for at least 6 months, and continued to express the CAR. T cells were effective in an anti-tumor response even at low dosage levels of about 1.5×10^5 cells/kg (45). The prolonged persistence of CD28-4-1BB-CD3ζ CAR modified T cells is probably due to two effects, the cooperation of costimulation in sustaining T-cell survival in the long-term and the repetitive re-stimulation by $CD19^+$ healthy B cells and their progenitors which are also targets for the anti-CD19 CAR T cells. Apart from grade-3 tumor lysis syndrome and a cytokine storm, T-cell infusions had no other acute toxic effects in that trial. Interestingly, there was a delayed increase in the pro-inflammatory cytokines IFN-γ and IL-6, which paralleled the clinical symptoms and coincided with the elimination of leukemia cells from the bone marrow. The clinical application of an IL-6 neutralizing antibody, noteworthy, reduced clinical manifestation of the cytokine storm. The same CAR is currently being evaluated in the treatment of pediatric $CD19^+$ acute leukemia with spectacular success, however, relapse of $CD19^-$ leukemia during therapy was also observed in one case (46). In previous trials, CAR T cells expanded less and objective tumor responses were modest although clearly documented in two out of three patients (47–50).

Despite recent success, two fatal serious adverse events occurred after infusion of CAR T cells, one of which is at least in part contributed to the CAR targeting specificity. "On-target off-organ" activation of the CAR T cells occurred in the NIH trial based on the fact that the targeted Her2/neu (ErbB2) is ubiquitously expressed on healthy tissues (50). The other adverse event after treatment of a

Table 1 | Recent adoptive cell therapy trials using CAR engineered T cells.

Target antigen	Disease	CAR signaling domain	ClinicalTrial.gov identifier	Clinical center
CD19	B-CLL	CD28-CD3ζ	NCT00466531	MSKCC
CD19	B-ALL	CD28-CD3ζ	NCT01044069	MSKCC
CD19	Leukemia	CD28-CD3ζ	NCT01416974	MSKCC
CD19	Leukemia/lymphoma	CD28-CD3ζ	NCT00924326	NCI
CD19	Leukemia/lymphoma	CD28-CD3ζ	NCT01087294	NCI
CD19	Leukemia/lymphoma	CD28-CD3ζ vs. CD3ζ	NCT00586391	BCM
CD19	B-NHL/CLL	CD28-CD3ζ vs. CD3ζ	NCT00608270	BCM
CD19	Advanced B-NHL/CLL	CD28-CD3ζ vs. CD3ζ	NCT00709033	BCM
CD19	ALL post-HSCT	CD28-CD3ζ	NCT00840853	BCM
CD19	Leukemia/lymphoma	CD137-CD3ζ	NCT01029366	UP
CD19	B-lymphoid malignancies	CD28-CD3ζ	NCT00968760	MDACC
CD19	B-lineage malignancies	CD28-CD3ζ	NCT01362452	MDACC
CD20	Mantle cell lymphoma/indolent B-NHL	CD28-CD137-CD3ζ	NCT00621452	FHCRC
PMSA	Prostate cancer	CD28-CD3ζ	NCT01140373	MSKCC
CEA	Breast cancer	CD28-CD3ζ	NCT00673829	RWMC
CEA	Colorectal cancer	CD28-CD3ζ	NCT00673322	RWMC
Her2/neu	Lung cancer	CD28-CD3ζ	NCT00889954	BCM
Her2/neu	Osteosarcoma	CD28-CD3ζ	NCT00902044	BCM
Her2/neu	Glioblastoma	CD28-CD3ζ	NCT01109095	BCM
Kappa light chain	B-NHL and B-CLL	CD28-CD3ζ vs. CD3ζ	NCT00881920	BCM

MSKCC, Memorial Sloan-Kettering Cancer Center; NCI, National Cancer Institute; BCM, Baylor College of Medicine; RWMC, Roger Williams Medical Center; UP, University of Pennsylvania; MDACC, M.D. Anderson Cancer Center; FHCRC, Fred Hutchinson Cancer Research Center.

$CD19^+$ CLL patient with CD28-CD3ζ CAR T cells was attributed to an extravasation of a latent bacterial infection subsequent to lymphodepletion (51). Despite the observed severe adverse events, MHC-independent targeting of cancer cells by CAR modified T cells showed promise in controlling $CD19^+$ leukemia in the long-term; currently initiated and future trials will address whether solid cancer lesions will also successfully be targeted and controlled by CAR T cells.

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