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Review Article

Dendritic Cells The Tumor Microenvironment and the Challenges for an Effective Antitumor Vaccination

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Many clinical trials have been carried out or are in progress to assess the therapeutic potential of dendritic-cell- (DC-) based vaccines on cancer patients, and recently the first DC-based vaccine for human cancer was approved by the FDA. Herewith, we describe the general characteristics of DCs and different strategies to generate effective antitumor DC vaccines. In recent years, the relevance of the tumor microenvironment in the progression of cancer has been highlighted. It has been shown that the tumor microenvironment is capable of inactivating various components of the immune system responsible for tumor clearance. In particular, the effect of the tumor microenvironment on antigen-presenting cells, such as DCs, does not only render these immune cells unable to induce specific immune responses, but also turns them into promoters of tumor growth. We also describe strategies likely to increase the efficacy of DC vaccines by reprogramming the immunosuppressive nature of the tumor microenvironment.

1. General Characteristics of Dendritic Cells

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) found in peripheral tissues and in immunological organs such as the thymus, bone marrow, spleen, lymph nodes, and Peyer's patches [1–3]. Their function is to scan peripheral tissues where they recognize, take up and process pathogens and present pathogen-derived antigenic peptides in the context of major histocompatibility molecules (MHCs) to naive T lymphocytes at lymphoid organs [4, 5]. Through these processes, DCs form a critical link between innate and adaptive immunity and are essential for the development of antigen-specific immune responses. To understand how DCs function in the development of adaptive immunity and the role of DCs in disease, one must first understand the distinguishing characteristics of innate and adaptive immunity.

Innate immunity is the first response to an immunological challenge, and the onset of an innate immune response is very rapid. Once a foreign pathogen breeches the outer barrier of the skin and enters the body, several innate immune cells are present to resolve this challenge. Some of the key immune cells that participate in the innate immune response include macrophages, granulocytes, DCs, and natural killer (NK) cells. Macrophages, along with granulocytes and DCs, are all phagocytic cells found in tissues. After taking up a pathogen, these phagocytic cells are able to eliminate it through several mechanisms such as reactive oxygen or nitrogen species. The means by which pathogens are detected by phagocytes is through the expression of conserved pathogen-associated molecular patterns (PAMPs) present on the cell surface of the pathogen. These PAMPs are detected by pattern recognition receptors (PRRs) expressed on the cell surface of the phagocyte. Through pathogen recognition by PRRs, the phagocytes of the innate immune response are able to distinguish between self and foreign (non-self) cells. Some of the main PRRs active in innate immunity include Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [6, 7].

2. DC Activation Process

Immature DCs present in peripheral tissues can detect foreign PAMP-bearing microorganisms through their high expression of cell surface and vesicular PRRs [8]. Following recognition, DCs take up pathogens by phagocytosis and process them into peptide fragments [3]. Since not all pathogens are eliminated by the innate immunity, an adaptive immune response may be needed to target antigenic epitopes associated with the pathogen to resolve the immunological threat completely. Antigenic peptide fragments derived from the processed pathogen are bound and presented on the DC surface by MHC molecules. These MHC molecules can evoke the adaptive immune response by presenting antigenic peptides to naïve T cell receptors [3].

An immature DC that has processed a pathogen will undergo maturation in the presence of proinflammatory cytokines and migrate to lymphoid regions where it can present the antigen peptide to naïve T lymphocytes [3, 4]. The maturation process involves upregulation of MHC class II molecules, costimulatory molecules such as CD40, CD80, CD86, and OX40L, and the chemokine receptor CCR7, while downregulating the expression of the chemokine receptor CCR6. Upon maturation, DCs show a decrease in their phagocytic capability, an augment in their efficacy to present processed antigens in the context of MHC molecules, and consequently an improved capability to activate T cells. Chemokines CCL19 (ELC) and CCL21 (SLC), ligands for CCR7, are constitutively expressed at high levels in lymph nodes [9]. Thus, mature DCs migrate from the sites of antigen capture to T-cell regions of draining lymph nodes, where they contact naïve or memory T cells and initiate a specific immune response [3, 10]. In this manner, DCs form the vital link between innate and adaptive immunity.

3. DCs Subsets in the Mouse

Murine DCs have been broadly divided into myeloid and plasmacytoid populations. The myeloid DCs, currently termed conventional DCs (cDCs), are further subdivided into several subsets present in immune and nonimmune tissues and organs specialized to perform different functions as described below. CD11c has been used as a typical marker of murine cDCs although additional markers have been used to distinguish these cells from other leukocytes such as NK cells and B cells that can also express it. Indeed, all cDC populations (except pre-DCs) are characterized by expressing high levels of CD11c [11, 12]. In the steady state cDCs present in lymphoid organs and tissues originated from bone marrow precursors. As extensively reviewed by Liu and Nussenzweig, 2010 [11], the mouse bone marrow harbors a common DC precursor (CDP) characterized by high expression of CD115 and Flt3, low expression of CD117 ($CD117^{lo}$), and is negative for lineage markers CD3, NK1.1, B220, TER-119, and Gr-1 (Lin^-) [13]. This precursor is derived from a common monocyte and DC precursor also present in the bone marrow [11, 13]. The CDP gives rise to a pre-DC circulating precursor ($CD11c^+MHCII^-SIRP\alpha^{lo}$) that rapidly reaches the lymphoid organs or tissues [11, 14]. As shown in Figure 1, two major DC subpopulations are present in mouse spleen in the steady state, $CD11c^{hi}MHCII^+CD8\alpha^+CD205^+SIRP\alpha^-CD11b^-$ and $CD11c^{hi}MHCII^+CD8\alpha^-33D1^+SIRP\alpha^+CD11b^+$ cells [11, 12]. As determined by elegant studies performed by Dudziak et al. 2007, [15], the $CD8\alpha^+$ DC subpopulation is specialized in cross-presentation, primarily presenting peptides associated with MHC-I antigens, while the $CD8\alpha^-$ subpopulation is involved in presenting MHC-II-associated peptides. It has been proposed that the $CD8\alpha^+$ splenic population exclusively expresses the chemokine receptor XCR1, thus being an excellent marker to investigate this subpopulation in other species [16]. It has been recently reported that this marker is also expressed by lymphatic resident and migratory $CD8\alpha^+$ DCs [17], suggesting a common origin for these cells. In addition, it has been shown that the transcription factor *Batf3* is selectively required for the development of $CD8\alpha^+$ DC subset [18]. Although cDCs were previously considered to be terminal mature cells, growing evidence has determined that around 5% of spleen DCs are actively diving at any given time [11, 12, 14, 19].

Similar $CD8\alpha^+$ and $CD8\alpha^-$ DC populations to the ones observed in mouse spleen are present in the lymph nodes and thymus [11, 12]. In addition a $CD11c^+MHCII^{hi}langerin^+CD40^{hi}$ DC migratory subpopulation has been detected at the level of lymph node and tissues [11]. cDC subpopulations have also been characterized as $CD11c^{hi}MHC^+CD103^+CD11b^-$ or $CD11c^{hi}$ MHC^+CD103^- $CD11b^{hi}$ in different organs such as the liver, lung, and kidney [11]. At the level of the intestine, cDCs are populating both Peyer patches ($CD11c^{hi}MHC^+$ $CD103^+CD11b^{lo}CX3CR1^-$ and $CD11c^{hi}MHC^+CD103^-$ $CD11b^{hi}CX3CR1^+$) and lamina propria ($CD11c^{hi}MHC^+CD$ 103^+ $CD11b^+CX3CR1^-$) [11]. Finally, the skin presents a particular subtype of DCs, the LCs, which are considered to be derived from a pre-LC precursor. These cells are characterized by expression of $CD103^+CD11b^{lo}langerin^+$ or $CD103^-CD11b^{hi}langerin^-$ (both in the dermis) and $CD11c^{hi}CD205^{lo}langerin^+EpCAM^{hi}$ (epidermis) [20]. It has been reported that epidermal Langerhans cells and langerin dermal DCs constitute the vast majority of skin DCs, while langerin dermal DCs represent 5% of all skin DCs [20].

Finally, conventional $CD8\alpha^+$ DC and $CD103^+$ DCs present in different nonimmune tissues express similar *Batf3* requirements, indicating that they might be closely developmentally related [21].

The other main subset of DCs is comprised by plasmacytoid DCs (pDCs). In the mouse, these cells, also derived from the CDP [14], are characterized by the expression of B220, CD45RB, low or null levels of CD11c, and no CD11b [22]. Circulating pDCs have the capability of producing large amounts of type 1 IFN in response to viral infections [22, 23] and so are key mediators of the innate immune response against viruses.

Different protocols have been developed in order to generate murine DC cultures. Usually, these cells are differentiated *in vitro* from bone marrow precursors using GM-CSF alone or in combination with IL-4 [24–26]. The use of

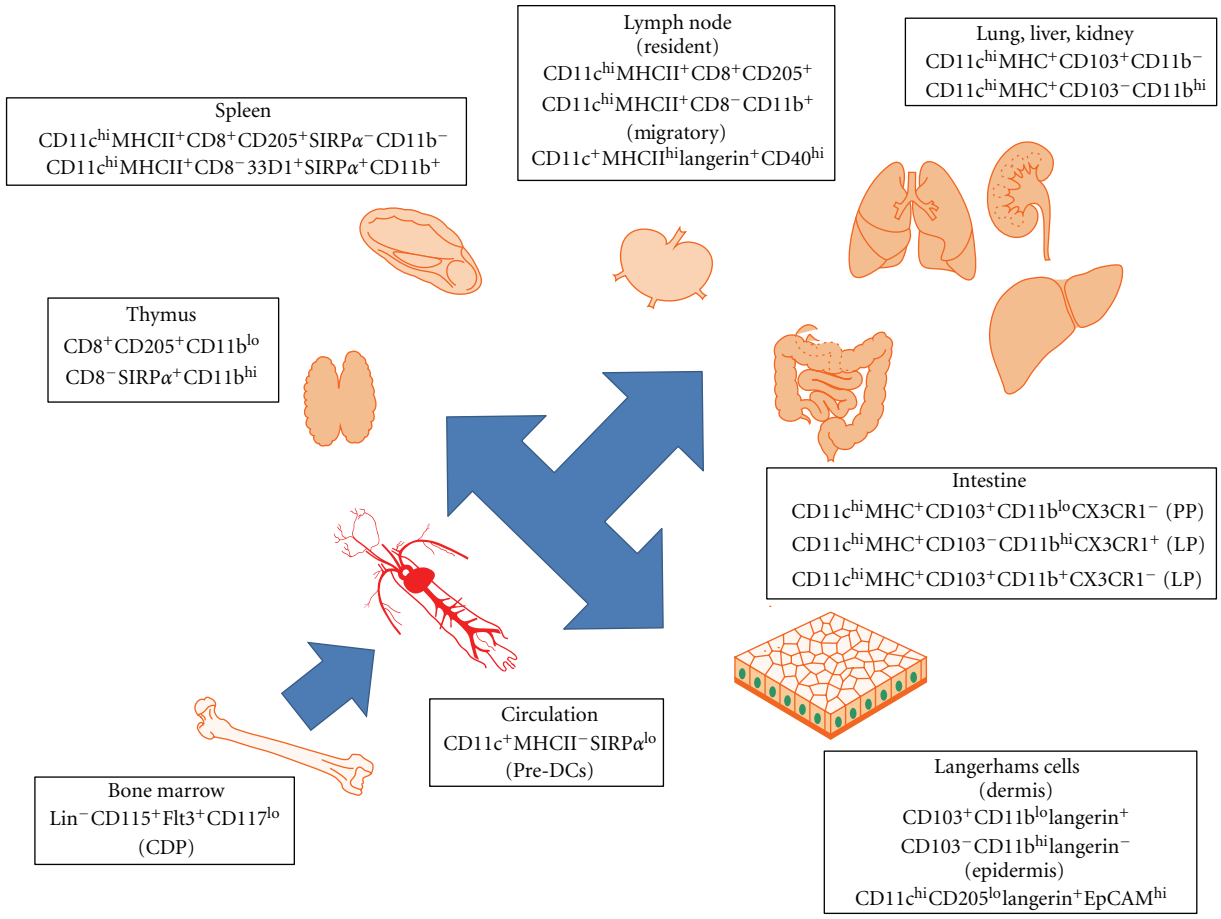

Figure 1: Conventional murine DCs in the steady state. Several DC subpopulations have been described in the mouse model colonizing lymphoid organs and other tissues. Figure adapted from Motifolio Biomedical Toolkit Suite.

GM-CSF and/or IL-4 generates high amounts of dendritic cells, capable of stimulating T cells *in vitro* and *in vivo*, which have been extensively used in order to investigate DC: T cell interactions, determine the efficacy of DC-based vaccines, and determine their role in pathological conditions such infectious diseases or tumor models [27–34]. Alternatively, *in vitro* generated DCs can be obtained from bone marrow progenitors by treatment with fms-related tyrosine kinase 3 ligand (Flt3) and cytokines such as IL-6, stem cell factor, IL3, or insulin-like growth factor [25, 26, 35]. The DC populations generated upon culture of these precursors with Flt3 have been considered to more closely resemble CD8α^{+} splenic DCs, particularly in their capability of producing IL-12 and or cross-present antigens, although lacking expression of CD8α [36]. Finally, Flt3 can be also used for expansion of murine DCs *in vivo* [35, 37].

4. Murine DC Subsets during Inflammation and Disease

It has been postulated that in the steady state murine DCs only originate from DC precursors, while during inflammatory or pathological settings they might also arise from monocytes and colonize lymphoid organs or nonimmune tissues [38–42]. In addition, it has also been demonstrated that, upon CD11c depletion, monocytes can contribute to DC repopulation at the level of the intestine [43]. Recent data has challenged this, suggesting that even in the steady state some DC populations can arise from monocytes [44]. In particular, as reported by Jakubzick et al., 2008 [45], in the absence of inflammation CD103^{+} and CD11b^{hi} pulmonary DCs can, respectively, originate from two different monocyte populations characterized by the high or low expression of Ly-6.

Nevertheless, particular DC populations are generated under inflammatory conditions. For example, it has been shown that a DC subset specialized in generating high levels of TNFα and upregulating nitric oxide synthase II is originated from monocytes during bacterial infections [40]. These TNF/iNOS-producing (Tip) DCs are recruited to the spleen via CCR2 signaling and have been shown to mediate the innate immune response against *Listeria monocytogenes*, an intracellular bacterial pathogen [38].

The generation of particular DC populations has also been observed in pathological conditions such as cancer. For example, a DC subset with cytotoxic activity has been described in the last years. This subset, named killer

DC, is characterized by coexpression of B220 and NK1.1 receptors and is able to kill tumor cells, thus preventing tumor growth when used in adoptive therapies [46–49]. These $B220^+CD11c^+NK1.1^+$ DCs produce large amounts of interferon γ (IFNγ) and are named IFN-producing killer DCs (IKDCs). *In vitro* studies using fusokines (molecules generated by fusing different chemokines) have shown that murine monocytes can be transformed into inducible killer DCs with the capability of inducing apoptosis of tumor cells without losing their antigen presenting capabilities [50]. In addition, treatment of bone marrow precursors with MHC-I peptides in the context of a ligand epitope antigen presentation system (LEAPS) is able to generate yet another DC population characterized by expression of levels of IL-12, thus being able to promote and steer immunity towards a specific T helper-1 (Th1) response [51, 52].

Another subset of DCs described in tumor settings is restricted to the spleen, express CD19, and suppresses T cells responses via indoleamine 2,3-dioxygenase (IDO) expression [53–56]. The expression of IDO in these cells is triggered upon CTLA4-mediated ligation of CD80 or CD86 molecules [53].

Adding to the complexity of DC subsets, it has been shown that some DC populations can change their phenotype under pathological settings. For example, pDCs could acquire cDC characteristics under the influence of viral infection [57]. This DC plasticity was evidenced by pioneering work showing that $CD8\alpha^-$ DCs can give rise to other splenic DC subpopulations [58].

5. DCs in Humans

Characterization of DC populations in humans is challenging due to their low numbers in circulation (less than 1% of blood mononuclear cells) and limited availability of healthy tissues as opposed to animal models. As in the mouse, human circulating DCs are broadly divided into pDCs and cDCs, characterized by expression of MHC-II and $CD11c^-CD123^+$ (plasmacytoid) or $CD11c^+CD123^-$ (conventional) antigens. cDCs have been further divided into those characterized by the expression of CD16, CD1c (BDCA-1), and CD141 (BDCA-3) [1, 59]. As described in detail by MacDonald et al., 2002 [59], the circulating cDC population was composed by 40%–80% of $CD16^+$ DCs, 20% to 50% of $BDCA1^+$ DCs, and 2% to 3% of $BDCA3^+$ DCs. Much effort has been put into determining the homology of these populations to murine $CD8\alpha^+$ and $CD8\alpha^-$ DC populations, although human cDCs do not express this marker. Recent reports indicate that $BDCA3^+$ DCs might be the putative homologues of murine $CD8\alpha^+$ DCs due to their expression of TLR-3, baft3 [60], and XCR1 [16, 17, 61], their capability of producing IL-12 upon stimulation [60], and their higher capability of cross-presenting antigen when compared to $CD16^+$ and $BDCA1^+$ DCs [60–62]. These DC populations can be also detected in human spleens [60]. On the contrary, these cells do not express TLR9 as their murine putative counterparts [60]. In addition, array analysis clustered together human $BDCA3^+$ with mouse $CD8\alpha^+$ and human $BDCA1^+$ with murine $CD8\alpha^-$ DCs [63].

Three different DC subsets have been described in human skin characterized by expression of $CD1a^{high}CD14^-$ $HLA\text{-}DR^+$, $CD1a^{dim}CD14^-HLA^-DR^+$ DCs, and $CD1a^-$ $CD14^+$ $HLA\text{-}DR^+$ DCs [64]. $CD1a^{high}CD14^-HLA\text{-}DR^+$ Langerhans cells reside in the epidermis, while the other subsets reside in the dermis but contrary to what happens in the mouse they do not express langerin [64].

Recently, 2 skin-derived and 2 resident human cDC subsets were described in skin-draining lymph nodes characterized by the expression of $CD1a^+CD11c^{int}$ $langerin^+E\text{-}cadherin^+$ (skin Langerhans cells); $CD1a^+CD11c^{hi}$ and variable expression of langerin contrary to what was described above (dermal Langerhans cells); $CD14^-BDCA3/CD141^{hi}$ $CD103^-$ and $CD14^+$ $BDCA3^{lo}CD103^+$ [65].

Finally, in order to generate high amount of DCs for vaccination purposes, these cells have been prepared *ex vivo* from monocytes or $CD34^+$ precursors [66–68].

6. DCs and T Cell Responses: The Four Signals

DCs play a multitude of roles in the development of an antigen-specific immune response. Through the expression of both MHC class I and MHC class II molecules, DCs are able to interact with and activate naïve $CD8^+$ T cytotoxic and naïve $CD4^+$ T helper lymphocytes, respectively [7, 10, 69]. For a naïve T lymphocyte to become an effector cell different signals are required. The first signal comes from the direct interaction of the T cell receptor (TCR) of the naïve T lymphocyte with the peptide bound to the MHC molecule (Signal 1). The second signal required for naïve T cell activation comes from DC: T cell interactions through costimulatory molecules such as CD80 and CD86 on the DC surface with CD28 on the T cell surface (Signal 2). If costimulatory signaling fails to occur, the T lymphocyte will not become activated and T cell anergy will ensue. The third signal derived from DCs, which can lead to a specific immune response, is T-cell differentiation through cytokine signaling (Signal 3). There are multiple T helper subsets, and the differentiation of naïve $CD4^+$ T helper cells into activated effector T helper cells is directed by DC-derived cytokines. Recently, it has been proposed that DCs give an additional signal to T cells [70]. This signal 4 instructs T cells to migrate to particular tissues by inducing the expression of specific chemokine receptors and integrins in these cells upon interaction with antigen-pulsed DCs [70].

Effective activation of T cells will depend in the end on the levels of expression and the interplay between positive and negative costimulatory molecules in both DCs and T cells. For example, antigen uptake in the absence of inflammatory signals renders phenotypically immature DCs, expressing low levels of MHC-II and costimulatory molecules. Importantly, antigen presentation in the absence of effective positive costimulation can lead to T-cell anergy and tolerance [71]. These DCs are considered "tolerogenic" in comparison to "immunogenic" DCs capable of inducing potent specific immune responses. Interestingly, DCs can switch from immunogenic to tolerogenic depending on the microenvironment conditions. For example, viral infections

can differentiate pDCs into T-helper-1- (Th1-) inducing DCs [57] while IL-3 can induce Th1-inducing DCs to differentiate into Th-2-inducing ones [72].

7. Properties of the Tumor Microenvironment

Tumors are composed not only by tumor cells, but also by other cellular types such as fibroblasts, endothelial cells, and infiltrating leukocytes that together with extracellular matrix components constitute the microenvironment of the tumor. In recent years the relevance of the tumor microenvironment as a key player in tumor development has been highlighted and the role of its different populations investigated. The protective role of the immune system against tumors has been widely described and tumor-infiltrating lymphocytes, for example, have been associated with improved survival of patients with melanoma, prostate, breast, colorectal, and ovarian carcinomas, among others [73–76]. On the contrary, tumor-associated leukocytes such as regulatory T cells (Treg) or myeloid-derived suppressor cells (MDSCs) can promote tumor growth by inhibiting antitumor immune responses [77, 78]. Indeed, we have previously demonstrated the relevance of the tumor microenvironment in attracting MDSCs by a complement-mediated process [79]. Further, in a tumor setting a subset of spleen DCs with the capability of suppressing T cells responses via indoleamine 2,3-dioxygenase (IDO) expression has been described [53].

In addition to suppressing the immune response, tumor-associated leukocytes can also promote angiogenesis. Leukocyte infiltration can precede the development of a neoplasm, with being chronic inflammation being an important risk factor for the development of cancer [80–82]. Indeed, inflammatory conditions such as those caused by certain types of infections can be involved in the pathogenesis of many human malignancies. For example, gastric carcinomas can arise in a *H. pylori*-induced gastritis environment [81] or hepatitis B virus/hepatitis C virus can induce hepatocellular carcinomas [82]. Also, chronic but noninfective inflammatory conditions as in the case of smoking-related bronchial cancer can induce carcinogenesis [83]. In the same way, chronic pancreatitis is considered a risk factor for the development of pancreatic cancer, and many of the growth factors involved in tissue remodeling and regeneration in chronic pancreatitis are present in pancreatic cancer [84]. In particular, infiltrating inflammatory cells secrete a diverse repertoire of growth factors and proteases that enhance tumor growth by stimulating angiogenesis. We and others have described the capability of antigen presenting cells such as DCs or macrophages, to collaborate with neoangiogenesis in human cancers and in different mouse tumor models [5, 85–89].

8. Characteristics of Tumor-Associated DCs

DCs are conspicuous members of the microenvironment of several types of cancer [86, 90–93]. Tumor-associated cytokines such as vascular endothelial growth factor (VEGF), interleukin- (IL-) 10, and prostaglandin E-2 (PGE2) can profoundly affect the nature of DCs [94]. Several reports indicated that tumor-associated DCs (TA-DCs) are immunosuppressive, incapable of inducing specific immune responses, or can induce regulatory T cell expansion. In particular, DCs showing low levels of costimulatory molecules have been detected in tumors expressing high levels of VEGF [95]. But besides an immune "paralysis," we and others have shown that TA-DCs, or leukocyte expressing DC markers, are able to produce angiogenic factors and can promote angiogenic processes in the tumor microenvironment [79, 86, 93, 96].

Tumors require blood supply for expansive growth. With increasing distance from vessels, hypoxic tumor cells produce angiogenic factors that induce the formation of neovessels [97–99]. Until recently, angiogenesis, or sprouting of endothelial cells from existing vessels, was the only accepted mechanism of tumor vascularization. Recent studies have suggested that vasculogenesis, or recruitment of endothelial progenitors that differentiate into endothelial cells, might contribute to the formation of tumor neovessels [100]. Endothelial cell progenitors were first identified by expression of the hematopoietic stem cell antigens, CD34 and flk-1, and other hematopoietic stem cell antigens, such as CD133 (AC133) [100]. Several populations of hematopoietic cells assume an endothelial phenotype when cultured under proangiogenic conditions. These include $CD34^+$, $Sca1^+$, $CD133^+$, and $CD14^+$ cells. In particular, the capability a $CD34^-$ monocytes to differentiate into endothelial-like cells *in vitro* has been reported [101–103]. Further, different studies have demonstrated that monocytes or monocyte-like cells can also function as endothelial cell progenitors and incorporate into growing vasculature in experimental models [104–106]. For example it has been recently shown that monocytes, under the influence of proteins present in the tumor microenvironment such as pleiotrophin or M-CSF, transdifferentiate into endothelial cells that incorporate into tumor blood vessels [107]. In addition, interaction of monocytes with extracellular matrix components such as fibronectin might also contribute to the monocyte-endothelial cell transdifferentiation process [108].

We and others have shown that DCs cultured in the presence of tumor factors can undergo an endothelization process characterized by the loss of CD14/CD45 and displayed endothelial markers such as CD31, CD34, von Willebrand factor, vascular-endothelial-growth-factor-receptor- (VEGFR-) 2, and VE-Cadherin [85, 109–112]. Furthermore, as we and others have shown, DCs can display other characteristics of endothelial cells such as LDL uptake, lectin binding, and formation of cord-like structures in 3D gels [85, 109, 110] and are able to assemble into vascular structures *in vitro* and *in vivo,* [85, 109, 110]. Although this evidence suggests that DCs can transdifferentiate into endothelial cells, the capability of these cells of acting as *bonafide* endothelial cells is debatable. For example, we have shown that tumor-associated DC precursors purified from mouse or human ovarian carcinomas are able to participate in the generation of neovessels *in vivo* [85, 88]. A follow-up study by Huarte et al., 2008 [113], demonstrated that these cells localize at the pericyte level *in vivo* in a mouse

model of ovarian carcinoma, acting as a scaffold for the generation of neovessels. Indeed, it has been shown that DCs have the capability of intimately interacting with endothelial cells and help to stabilize newly expanded vasculature at the level of lymph nodes [114]. Thus, it is tempting to speculate that in some tumor settings, this pericyte-like function of DCs might help shape the characteristics of tumor endothelium. In addition, DCs can also contribute to angiogenesis by producing factors that promote growth of *bonafide* endothelial cells [115].

9. Dendritic Cells as a Source of Angiogenic Factors

We have recently shown that myeloid DCs are able to produce a gamut of angiogenic molecules *in vitro* such as matrix metalloproteases, VEGF, angiogenin, heparanase, and basis fibroblast growth factors [116]. We have also previously shown that DC precursors participate in tumor progression and angiogenesis in a mouse model of ovarian cancer [85]. For those studies, we used the *ID8-Defb29/Vegf-A* mouse model of ovarian carcinoma. ID8 is a cell line derived from spontaneous *in vitro* malignant transformation of C57BL/6 mouse ovarian surface epithelial cells that we engineered to express mouse β-defensin 29 and VEGF-A. Our published data support that this model mimics the pathophysiology of human ovarian cancer which expresses both β-defensins and levels of VEGF-A similar to our model. *ID8-Defb29/Vegf-A* tumor cells are able to generate solid tumor or ascites when injected into syngeneic C57BL/6 mice subcutaneously or via the intraperitoneal route respectively. In this tumor model, immature DCs contribute to ovarian cancer progression by acquiring a proangiogenic phenotype in response to VEGF via VEGF-R2 [88, 115, 117]. Further, it has been shown that depletion of TA-DCs *in vivo* reduces tumor growth and decreases angiogenesis in this mouse model of ovarian cancer [113, 118]. In the same way, data from the late Dr. J. Folkman's lab [119] highlighted the contribution of DCs to angiogenesis in a murine model of endometriosis and in the peritoneal Lewis lung carcinoma tumor model. Similar to what we observed in our model, they showed that these proangiogenic DCs have an immature phenotype, and express VEGF-R2.

Taking into account all these data, it becomes clear that tumors have the capability to attract and reprogram the biology of DCs, inducing them to exert immunosuppressive or angiogenic functions.

10. Dendritic Cells and Antitumor Therapy

Considerable effort has been made in order to develop strategies for using DCs to induce tumor-specific immunity, including nearly 100 clinical trials designed to evaluate their safety or efficacy in humans [120]. The goal of DC-based vaccination for antitumor therapy is to stimulate robust and long lasting specific CD4 and CD8 T cell responses [121]. To accomplish this, several studies have been performed in order to generate DCs with the capability of inducing robust T cell responses. For vaccination studies, DCs have been generated from bone marrow precursors in the mouse and mostly from monocytes in humans as described above. Different steps in the antigen presentation process have been evaluated such as antigen loading, DC maturation, and delivery route and dose scheme.

Assayed methods of loading DCs with tumor-associated antigens in the mouse model included pulsing the cells with peptides derived from tumor antigens [122], whole tumor lysates [123], apoptotic or necrotic cells [124] alone or conjugated with toll-like receptor ligands [125], or antigens coated with antibodies to target them to DCs via Fcγ receptors [126]. We have showed that inducing the expression of danger signals in tumor cells by means of replication-deficient or replication-restricted virus appears to be an efficient method to pulse DCs for vaccination purposes [124]. In addition, other strategies include encapsulating peptides in biodegradable polymers that are phagocytized by DCs [127], preparing DCs fused with tumor cells [128], or pulsing DCs with RNA encoding tumor antigens [129]. In recent years, the use of lentiviruses to induce stable transduction of DCs has also been successfully evaluated [130–132]. These vectors have the advantage of infecting nondividing cells, thus being excellent tools to express different molecules in DCs. Moreover, hematopoietic stem cells have been transduced with lentiviruses and then differentiated into antigen-expressing DCs [133].

Similar studies have been performed using human DCs. Among other strategies, these cells have been pulsed *in vitro* with apoptotic or necrotic cells [134, 135], with nucleic acids encoding tumor antigens [136, 137], or fused with tumor cells [138] or pulsing DCs. An alternate method for loading DCs with tumor antigen involves the insertion of full-length antigens by genetic modification using viral systems. Vector-transferred recombinant antigens synthesized in the cytosol of the cells may enter the degradation process of intracellular molecules, yielding peptides that can be directly presented by MHC-I molecules. Several viral vectors have been used to transduce human DCs [139] including recombinant adenoviruses [140–142], poxviruses [143], and retroviruses [139]. Lentiviruses have also been used to induce stable transduction of human hematopoietic stem cells or DCs [144, 145].

This information regarding DC pulsing have been translated to the human, where clinical trials have involved, among others, DCs pulsed with peptides [146], whole-tumor lysates [147], with RNA encoding tumor antigens [27, 148, 149], or fused with tumor cells [150, 151].

In order to improve DC-based vaccines for human therapy, different methods to induce DC maturation and optimization of antigen processing and presentation have also been proposed [121, 152]. The most widely used maturation protocol for human monocyte-derived DCs employs the combination of IL-6, tumor-necrosis-factor- (TNF-) α, IL-1β, and PGE-2. Although these *ex vivo* matured DCs have the capability to migrate towards lymph nodes, PGE-2 has been shown to induce the production of IL-10 and VEGF, which can be harmful in a tumor setting. Moreover, these maturation stimuli have been shown to generate mature DCs

capable of expanding regulatory T cells *in vitro* and *in vivo* [153, 154]. Alternative protocols using different TLR ligands have been extensively studied [155–157].

In recent years a different maturation cocktail has been tested on human DCs. This cocktail, named the αDC1 cocktail, is composed of a combination of cytokines and TLR ligands (IL-1β/TNFα/IFNα/IFNγ/poly-I:C) [156, 158, 159]. The αDC1 cocktail has been suggested as a better option for maturation since treated DCs show higher migratory responses to SLC, a CCR7 ligand constitutively produced by lymph nodes, and produce higher levels of IL-12p70 as compared to DCs matured with TNF-α, IL-1β, and PGE-2 [155]. But some data argues that this cocktail does not induce better T cell activation [160]. Other proposed maturation strategies for human DC vaccines involve activating tumor antigen-pulsed DCs with CD40 ligand before injection [161].

As previously reviewed in detail [162, 163], clinical trials with DC vaccines have used different methods of antigen pulsing, maturation status of the cells, route of administration, and dose scheme. The use of so many different strategies makes it difficult to interpret in detail the causes for the success or failure of the vaccinations, and argues for a consensus regarding DC preparation, maturation, and route and dose scheme for DC-based vaccinations.

11. The First FDA-Approved Antitumor DC Vaccine and the Challenges for Improvement

Recently, the first autologous cellular vaccine for antitumor therapies (Sipuleucel-T) has been approved by the Federal and Drug Administration (FDA) for the treatment of asymptomatic or minimally symptomatic metastatic castrate-resistant (hormone refractory) prostate cancer. In order to generate the vaccine, the patients are subjected to apheresis and the cells are cultured for 36–44 h in a media containing a synthetic protein generated by the fusion of prostatic acid phosphatase and GM-CSF [164]. Then, this preparation containing at least 50 million $CD54^+$ antigen-presenting cells is infused back into the patient. In all, the process may consist of three cycles of apheresis, pulsing stimulation, and reinfusions [164]. Sipuleucel-T therapy has shown mild to moderate, short-term, reversible adverse events in patients with no evidence of a treatment-related increase in autoimmune complications or secondary malignancies [165]. The treatment generates an increase in patient survival. In particular, it has been reported in a clinical trial that the median survival in treated prostate cancer patients was 4.1 months longer (25.8 months) than in the placebo group (21.7 months) [166]. Taking into account the promising but rather modest increase in the patients' survival, it becomes clear that efforts must be done in order to improve the efficacy of DC-based vaccines. A recent clinical trial in human ovarian carcinoma shows that DC vaccine therapy induced an increase in the antitumor immune response in treated patients [167]. Interestingly, an impaired immune response against an unrelated vaccine antigen in the same patients highlights the immunosuppressive status induced by tumors [167]. Thus, in order to increase the efficacy of these vaccines it might be interesting to block at the same time the deleterious influence of the tumor microenvironment. The role of the tumor microenvironment is also relevant, taking into account that most human DC vaccines are generated from monocytes, which, as described above, have a high plasticity and can change their phenotype in response to tumor factors.

12. Reprogramming the Tumor Microenvironment to Enhance DC Vaccination Efficacy

In general, although several reports indicate that DC vaccines are able to induce immune responses in cancer patients, they have only rarely resulted in objective clinical responses based on the response evaluation criteria in solid tumors (RECISTs) and no indication or evidence has been obtained that DC vaccines represent a method of stimulating protective immunity in cancer patients that is superior to other vaccination strategies [121]. One of the main reasons why DC vaccines have been suboptimal in clinical trials might be the inhibitory effect of the tumor microenvironment. As described above, the tumor microenvironment is highly immunosuppressive due the presence of soluble factors such as VEGF or IL-10 and immunosuppressive cell populations such as MDSCs and Treg. This can affect the efficacy of DC vaccination at different levels. First, there could be a direct effect of soluble factors on DC-based vaccines, impairing their immune capabilities. Indeed, it has been shown in a clinical trial that *ex vivo* matured DCs, loaded with tumor antigen, could be trapped by the tumor microenvironment, thus rendering the immunization completely ineffective [168]. Although this argues for an intranodal immunization with DC vaccines, factors produced by the tumor microenvironment can affect distal tissues [169]. For example, VEGF, which is produced by several tumors, can modify the immunological profile of lymph nodes, and the generation of immune precursors at primary and secondary lymphoid organs [95, 170]. Thus, not only TA-DCs might be affected by the tumor microenvironment. In addition, tumor-induced Treg and MDSCs can directly impair the antitumor properties of T effector cells induced by DC vaccines. Taking into account this, in the last years different strategies have been proposed in order to improve the efficacy of DC vaccines by reprogramming the immunosuppressive status of the tumor microenvironment.

The most common strategy has involved the combination of DC vaccination with Treg depletion. To accomplish this, Tregs have been depleted *in vivo* by antibody therapy with anti-CD25, a molecule expressed at high levels by Tregs. In the mouse, promising results were obtained in different tumor models, where it was reported that Treg depletion enhanced the efficacy of the vaccination [171–173]. In clinical trials, it has been shown that depletion of Tregs

before vaccination with DCs pulsed with carcinoembryonic antigen (CEA), enhanced specific T-cell immunity against CEA in patients having a metastatic cancer expressing CEA, as defined by immunohistochemical analysis or elevated CEA in peripheral blood [174]. In addition, depletion of Tregs also enhanced antitumor immunity in patients harboring metastatic renal cell carcinomas [175]. In both trials, Tregs were depleted by using denileukin diftitox, a fusion between the active domain of diphtheria toxin and IL-2 that binds cells expressing high levels of CD25. Upon internalization, this molecule leads to cell death due to blockade of protein synthesis. On the other hand, Jacobs et al., 2010 [176], demonstrated in a phase I/II study in metastatic melanoma that depletion of Tregs with daclizumab, a humanized antibody directed against CD25, did not enhance antitumor immunity in treated patients. These data point out that the efficacy of this combinatorial therapy might depend on the type of cancer and the strategies used to deplete the Treg population prior to vaccination.

A novel proposed combinatorial strategy assayed in the mouse model involved adding the use of CTLA-4 blockade to Treg depletion in the context of DC-based vaccination [177]. In this study, CTLA-4 blockade and depletion of Treg cells improved the potency of DC vaccination in a mouse model colon carcinoma expressing both CEA and HLA-A2 antigens.

Other proposed strategies involve elimination of tumor cells by radiotherapy, chemotherapy, antibody therapy, or viral oncolytic therapy in combination with DC vaccination as determined in animal models of lymphoma and prostate cancer, among others [178–181]. This aims to decrease the deleterious effect of tumor cell products while generating an inflammatory milieu that can enhance the efficacy of the vaccination. TA-DCs are usually described as immature cells with low expression of costimulatory molecule and, incapable of inducing robust antitumor immune responses [182–185]. In this way, elimination of cancer cells will not only generate tumor antigen that can be acquired by resident DCs, but also abrogate the immunosuppressive milieu generated by molecules produced by the same cancer cells. In this new milieu, DCs that have acquired tumor antigen might be able to turn into mature DCs, thus being able to induce effective immune responses.

Interestingly, depletion of TA-DCs from the tumor microenvironment of ovarian cancer has been shown to boost antitumor immune responses in the mouse model [113]. Thus, depletion of these cells prior to DC-based vaccination may induce effective antitumor immune responses. Finally, albeit generating a specific immune response, vaccination efforts may fail due to the incapability of T cells to reach their targets. Indeed, it has been previously shown that differential expression of endothelin receptor B in murine tumor endothelial cells determines the capability of T cells of infiltrating tumors [186]. Thus, future strategies designed to reprogram the tumor microenvironment to enhance the efficacy of DC vaccination might include blocking endothelin receptor in endothelial cells to facilitate cytotoxic T cell infiltration into the tumors.

13. Reprogramming DCs In Situ to Induce Better Antitumor Immunity

As described above, DCs are present in the microenvironment of different tumors, but they are usually cells with impaired capability of inducing antitumor effector T cells. A tantalizing strategy would be to reprogram these cells *in vivo,* transforming them into effective antigen-presenting cells. Different strategies are being developed and have been assayed in the mouse model in order to specifically target DCs *in situ*. For example, targeted delivery of antigens to DCs via specific molecules expressed on the surface of these cells has been investigated. Targeting ovalbumin to CD205 and 33D1 molecules on the surface of DCs *in vivo* helped identify the antigen presenting properties of $CD8\alpha^+$ and $CD8\alpha^-$ DC subpopulations of splenic DCs [15]. Building on these studies, effective immunization procedures have been obtained by using antibody-tumor antigen fusion proteins targeting DCs via CD205 [187] or CD11c [188]. In addition, antibodies specific for DC molecules have been used to coat liposomes or nanoparticles in order to deliver antigens and inflammatory compounds to DCs *in situ* in the mouse model [189] or to target human DCs [190]. Other strategies involve generating DC vaccines that express tumor antigens under a specific DC promoter, such as CD11c variant [191], or engineering antigen-carrying lentiviral vectors capable of selectively binding to DCs [192].

In the context of a murine ovarian cancer model, pioneering research has been performed by the Conejo-Garcia group [193] in order to reprogram DCs *in situ*. By using a mouse model of ovarian cancer, this group was able to demonstrate that *in situ* activation of TA-DCs can induce a potent antitumor immune response, creating a *de facto* vaccine with these cells. In order to accomplish this, they reprogrammed TA-DCs by administration of linear polyethylenimine nanoparticles encapsulating nonviral siRNA. These particles were avidly engulfed by TA-DCs, activating them through TLR5 and inducing a potent antitumor immune response. This strategy has the advantage of using the TA-DCs, which might already harbor tumor antigens [194]. If translatable to humans, this will avoid costly *ex vivo* preparation and pulsing of the patient's DCs. This *in situ* reprogramming of TA-DCs will benefit by combinatorial therapies destined to abrogate the immunosuppressive properties of the tumor microenvironment, such as using Treg depletion therapies.

14. Summary and Outlook for Future Development

Herewith we described that DCs comprise a population of leukocytes with the capability of inducing specific immune responses. These cells have the ability to capture antigens and select and activate T cells capable of recognizing and orchestrating an attack against the microbes or cells that harbor the same antigen. This property had made DCs ideal candidates for cellular vaccine therapies. DCs are divided into different subsets extensively investigated in the mouse

model. In recent years a similar complexity has started to unravel in humans. This heterogeneity is subjacent to a characteristic that seems to be a hallmark of these cells: their plasticity. It has been shown that these cells can modify their phenotype in response to microenvironmental factors. This characteristic seems to be exploited by tumors that not only repress the maturation of these cells, thus abrogating specific antitumor immune responses, but also transform them into promoters of angiogenesis. In the mouse model, it has been shown that DC-based vaccines can effectively induce antitumor immune responses. In humans, a cellular DC-based vaccine has been recently approved by the FDA for treatment of prostate cancer. In order to build on this promising scenario, combinatorial therapies destined to abrogate the deleterious influence of the tumor microenvironment are being investigated. This will render more powerful DC vaccines with the capability of generating a robust and long-lasting antitumor immune response.

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