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STATE-OF-THE-ART OF REGULATORY DENDRITIC CELLS IN CANCER

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Abstract

Dendritic Cells (DCs) with robust immunosuppressive activity are commonly found in the microenvironment of advanced solid tumors. These innate immune cells are generically termed regulatory DCs and include various subsets such as plasmacytoid, conventional and monocyte-derived/inflammatory populations whose normal function is subverted by tumor-derived signals. This review summarizes recent findings on the nature and function of regulatory DCs, their relationship with other myeloid subsets and unique therapeutic opportunities to abrogate malignant progression through their targeting.

Keywords

Dendritic Cell; tumor immunology; immunosuppression; emergency myelopoiesis; tumor microenvironment

1. INTRODUCTION

In the tumor microenvironment, DCs have a pivotal role for the activation of T cell-mediated anti-tumor immunity. However, in individuals with advanced cancer, tumor-derived extracellular inflammatory signals alter hematopoietic differentiation within the bone

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CONFLICT OF INTEREST STATEMENT

All the authors declare no conflict of interest.

marrow, resulting in an increase in myeloid output, termed “emergency” myelopoiesis. Altered myelopoiesis progressively impairs the capacity of DCs to prime and sustain immune protection against tumor growth, resulting in significantly accelerated malignant progression.

Recent mechanistic insight from different groups has unveiled important clues on how tumor-induced signals block the generation of mature, immunocompetent DCs in some tumors, while also transforming tumor-associated DCs (tDCs) from a potentially immunostimulatory to an immunosuppressive (regulatory) cell type in others. This review will focus on the role of regulatory DCs in advanced malignancies and how a better understanding of their activities and the immunosuppressive mechanisms that they elicit could lead to the design of more effective therapeutic interventions to restore anti-tumor immunity.

2. THE NATURE OF REGULATORY DCs IN THE TUMOR MICROENVIRONMENT

Although terminal differentiation of at least murine myeloid cells is impaired by tumor-derived factors, including VEGF-A (D. Gabrilovich, et al., 1998), rare populations of DCs with significant antigen-presenting capabilities have been identified in a variety of tumors for both humans and mice (Broz, et al., 2014). However, DCs spontaneously lacking the capacity to effectively stimulate T cells are a more common occurrence in the microenvironment of advanced solid tumors (Shurin, Ma, & Shurin, 2013). Besides losing their immunostimulatory function, tDCs also acquire direct inhibitory activities against tumor-reactive T cells and/or the ability to promote the generation of regulatory T cells (Treg). These DCs are generically termed regulatory DCs and include very diverse myeloid subsets of different origins and activities. Although the abundance and phenotype of regulatory DCs reported in different tumors varies, immunosuppressive attributes have been associated with DCs representing the major DC subsets in mice and humans: Plasmacytoid, conventional and inflammatory DCs (Bachem, et al., 2010; Haniffa, et al., 2012; Manh, Alexandre, Baranek, Crozat, & Dalod, 2013; Merad, Sathe, Helft, Miller, & Mortha, 2013; Segura & Amigorena, 2013, 2014; Segura, et al., 2013).

Plasmacytoid DCs (B220⁺CD11c^{low}MHC-II⁺ leukocytes co-expressing CD303 in humans and CD317 in mice) represent a rare DC subset specialized in the production of type I IFN in inflammatory conditions. They have been found to promote immunosuppression by enhancing IL-10 secretion by CD4⁺Foxp3⁻ T cells in human hepatocarcinoma (Pedroza-Gonzalez, et al., 2015), while their presence is associated with regulatory Th2 responses in breast cancer patients (Ghirelli, et al., 2015) and human melanoma (Aspord, Leccia, Charles, & Plumas, 2013), as well as Treg activity in human breast cancer (Sisirak, et al., 2013). Finally, plasmacytoid DCs contribute to tumor-induced immunosuppression by inducing CD8⁺ regulatory T cells in the human ovarian cancer microenvironment (Wei, et al., 2005).

Besides plasmacytoid DCs, other authors have identified tumor-induced regulatory DCs in preclinical mouse models as conventional DCs (recently identified as *Zbtb46*⁺ cells (Meredith, et al., 2012; Satpathy, et al., 2012)) co-expressing CD11c, MHC class II and co-

stimulatory CD80 and CD86 molecules and high levels of CD11b (Liu, et al., 2009; Norian, et al., 2009). Lin⁻ZBTB46⁺MHC-II⁺CD141⁺ conventional DCs (or their CD8a⁺CD4⁻ murine counterparts) typically cross-present extracellular antigens to CD8⁺ T cells, while Lin⁻ZBTB46⁺MHC-II⁺CD1c⁺ conventional DCs (or their CD8a⁻ counterparts in mice) express a distinct TLR pattern (Said & Weindl, 2015) and are particularly effective at inducing Th2 responses (O’Keeffe, Mok, & Radford, 2015). In cancer, however, CD11b⁺ conventional DCs have been found to suppress CD8⁺ T cell function in tumor-bearing mice, among other potential mechanisms, via L-Arginine metabolism (Norian, et al., 2009).

The phenotypic diversity of tumor microenvironmental DCs, however, cannot be understood without considering the pathological nature of myelopoiesis in cancer-bearing hosts. Thus, tumors are not only promoting inflammation at tumor beds, but also systemically, activating emergency myelopoiesis in bone marrow precursors. tDCs therefore include the short-lived leukocytes that typically arise from a hematopoietic lineage distinct from other leukocytes (Merad, et al., 2013; Schraml, et al., 2013), but also inflammatory cells derived from monocytes or DCs exhibiting markers differing from DCs generated during steady-state conditions, such as FcεRI and CCR7. Exhaustive studies by Amigorena and colleagues have characterized inflammatory DCs as human

CD11c⁺CD115⁺CD1c⁺CD1a⁺FcεRI⁺CD206⁺CD172a⁺CD14⁺CD11b⁺ leukocytes and CD11c⁺MHC-II⁺CD11b⁺F4/80⁺Ly6C⁺CD206⁺CD115⁺CD107b⁺FcεRI⁺CD64⁺ cells in mice (Segura & Amigorena, 2013, 2014). Although their precise ontogeny in inflammatory conditions remains unclear, inflammatory DCs can certainly arise from monocytes, and produce high levels of the pro-inflammatory cytokines TNF, IL-6, and IL-12 (O’Keeffe, et al., 2015).

Distinguishing macrophages and DCs in the tumor microenvironment (TME), however, is more complex than in the steady-state conditions because they arise from a continuum of pathological myelopoietic differentiation and receive conflicting signals at tumor beds. Although characterizing the heterogeneity of myeloid subsets in most human tumors remains a challenge, technical issues hampering the categorization of DCs vs. other myeloid subsets have been alleviated in recent years by the identification of the transcription factor ZBTB46 in *bona fide* conventional DCs, but not plasmacytoid DCs or other immune lineages (Meredith, et al., 2012; Satpathy, et al., 2012). It should be noted, however, that inflammatory cytokines induce *Zbtb46* expression in mouse monocytes (Poltorak & Schraml, 2015; Satpathy, et al., 2012). Despite inflammation-induced expression of *Zbtb46* in monocytes, the presence of DNGR1 (or proof of previous expression as determined by fluorescent reporters) has been established as an additional distinctive marker of the DC lineage (Schraml, et al., 2013).

Elucidating the ontogeny of tumor-infiltrating inflammatory myeloid cells is important because we have recently identified that CD11c⁺MHC-II⁺Dngr1⁺ cells expressing *Zbtb46*⁺ at much higher levels than splenic macrophages represent the most abundant myeloid subset in the microenvironment of different transplantable and autochthonous ovarian tumor models (Tesone, et al., 2016). These cells co-express the inflammatory DC markers FcεRI, CD11b and CCR7. Despite their abundance, expression of the DC lineage marker Dngr1 suggests that they are ontogenetically true DCs. These data challenge the notion that

inflammatory DCs always derive from monocyte precursors, although monocyte-derived DCs are clearly present in the microenvironment of different tumors. Importantly, the human counterpart of inflammatory DCs, characterized by concurrent expression of CD1c, CD11c, MHC-II and the absence of the B cell marker CD19 (Segura & Amigorena, 2013; Segura, et al., 2013) is also universally found in solid tumor masses from patients with ovarian cancer (Tesone, et al., 2016). Similar to murine tDCs, these cells represent *bona fide* DCs, as supported by greater expression of *ZBTB46* than autologous DCs in peripheral blood.

Unlike conventional regulatory DCs found in some preclinical models, DCs in the ovarian cancer microenvironment show relatively high levels of MHC-II, CD70 and CD86. Surprisingly, CD1c⁺CD11c⁺ DCs sorted from dissociated human tumors are not able to induce the proliferation of allogenic T cells, and their murine counterparts are able to effectively suppress T cell responses through a variety of mechanisms described in detail below. Hence, different tumors accumulate diverse subsets of DCs from various origins, which have in common their capacity to suppress T cell-dependent protective responses and therefore contribute to accelerate malignant progression.

3. TUMOR-INDUCED MECHANISMS CAUSING DC DYSFUNCTION IN CANCER

3.1. Pathological myelopoiesis is a hallmark of advanced malignancies that contributes to abrogate DC immunostimulatory function

Although most tDCs have the capacity of suppressing T cell responses (J. R. Cubillos-Ruiz, et al., 2009; Huarte, et al., 2008; Scarlett, et al., 2009), our studies have demonstrated that, at least in ovarian cancer, various stimuli that include the activation of TLR and CD40 signaling can promote the capacity of DCs to effectively present the antigens naturally captured in the TME, both *in vivo* and *in vitro* (Baird, et al., 2013; J. R. Cubillos-Ruiz, et al., 2012; J. R. Cubillos-Ruiz, et al., 2009; Scarlett, et al., 2009). To understand the mechanisms that drive APCs malfunction in tumors, it is important to first underscore that pathological myelopoiesis is a hallmark of virtually all advanced solid cancers. Tumors co-opt the mechanism of “emergency myelopoiesis” whereby effector leukocytes of the myeloid lineage are rapidly expanded to confront viral or bacterial infections in response to inflammatory cytokines (Takizawa, Boettcher, & Manz, 2012). Because established tumors promote both local and systemic inflammation, immature myeloid progenitors accumulate at lymphatic and tumor locations (D. I. Gabrilovich, Ostrand-Rosenberg, & Bronte, 2012; Ostrand-Rosenberg & Sinha, 2009). In most tumor-bearing hosts, myeloid leukocytes are retained at immature stages of differentiation by tumor-induced signals, which inhibit differentiation into committed DCs. Such signals include VEGF-A (D. Gabrilovich, et al., 1998) but also tumor-induced STAT3 signaling, which impairs DC generation by decreasing PKCβII abundance (Farren, et al., 2014). In addition, bone marrow-derived myeloid progenitors are influenced in the periphery of cancer-bearing hosts by inflammatory signals that are incompletely understood, eventually transforming them into immunosuppressive cells that are phenotypically different from immature myeloid cells developed during infection. When MDSCs from tumor-bearing mice are transferred into congenic tumor-bearing hosts, most of them turn into macrophages. However, ~5% of them differentiate at

tumor beds into cells with phenotypic attributes of DCs (Corzo, et al., 2010). Although the immunosuppressive nature of these MDSC-derived DCs remains to be determined, pathologically expanded DC precursors therefore are a likely cause of the accumulation of regulatory DCs in the TME.

3.2. Sustained Endoplasmic Reticulum (ER) stress responses promote DC dysfunction in the tumor microenvironment

Aggressive cancers thrive under hostile conditions such as hypoxia, nutrient starvation and oxidative stress by adjusting their protein folding capacity via the ER stress response pathway (Hetzel, Chevet, & Harding, 2013). The serine/threonine-protein kinase/endoribonuclease IRE1 α is the most evolutionarily conserved branch of this signaling pathway. Activated during periods of ER stress (e.g. accumulation of misfolded proteins in this organelle), the IRE1 α endoribonuclease domain excises a 26-nucleotide fragment from the *Xbp1* mRNA to generate a spliced variant that codes for the functional transcription factor, XBP1 (Yoshida, Matsui, Yamamoto, Okada, & Mori, 2001). This multitasking protein promotes cell survival by inducing expression of a broad range of critical genes involved in protein folding and quality control (Lee, Iwakoshi, & Glimcher, 2003).

While persistent XBP1 activation has been shown to facilitate malignant progression by promoting cancer cell survival and metastatic capacity under hypoxic conditions (Chen, et al., 2014), we recently identified another tumor-promoting function of aberrant IRE1 α -XBP1 signaling: by impeding the development of protective anti-tumor immunity in ovarian cancer via manipulation of normal DC function (J. R. Cubillos-Ruiz, et al., 2015). DCs residing in human and mouse ovarian cancers exhibited robust and sustained IRE1 α -XBP1 activation and concomitant overexpression of XBP1-dependent genes involved in the ER stress response (J. R. Cubillos-Ruiz, et al., 2015). Interestingly, abundant reactive oxygen species (ROS) found in tDCs promoted intracellular lipid peroxidation and the generation of reactive byproducts that triggered ER stress by directly modifying key ER-resident chaperones (J. R. Cubillos-Ruiz, et al., 2015). Accordingly, treatment with antioxidants or drugs that sequester lipid peroxidation byproducts impeded ER stress and IRE1 α -XBP1 activation in DCs exposed to tumor-derived factors like those commonly present in malignant ovarian cancer ascites (J. R. Cubillos-Ruiz, et al., 2015). Ovarian cancer-bearing mice selectively lacking XBP1 in DCs demonstrated delayed progression of primary and metastatic ovarian tumors in various preclinical models of disease (J. R. Cubillos-Ruiz, et al., 2015), and these effects were associated with the emergence of activated, antigen-experienced T cells producing IFN- γ in the tumor microenvironment (J. R. Cubillos-Ruiz, et al., 2015). Global profiling of tDCs revealed that XBP1 not only promoted the expression of regular XBP1-target genes mediating the ER stress response, but also induced a robust transcriptional lipogenic program leading to uncontrolled lipid accumulation (J. R. Cubillos-Ruiz, et al., 2015).

Pioneering studies by the group of D. Gabrilovich had previously unveiled that a major mechanism contributing to DC malfunction in cancer is indeed abnormal intracellular lipid accumulation. This dyslipidemia was shown to inhibit the efficient loading of antigenic peptides onto MHC-I molecules, thereby impairing optimal antigen cross-presentation to T

cells by DCs (Herber, et al., 2010). Consistent with this notion, XBP1-deficient tDC unable to accumulate intracellular lipid droplets demonstrated enhanced antigen-presenting capacity *in vitro* and *in vivo*, and tumor-reactive T cells generated in ovarian cancer-bearing mice lacking XBP1 selectively in DC demonstrated improved anti-tumor capacity when transferred into wild-type ovarian cancer hosts (J. R. Cubillos-Ruiz, et al., 2015). Our study therefore provided the first example of tumor cells co-opting IRE1 α -XBP1 signaling in DCs within the tumor microenvironment as a strategy to evade immune control.

3.3. Unremitting overexpression of Satb1 transforms DCs into inflammatory, immunosuppressive, tumor-promoting cells

Besides abrogation of antigen-presenting function, we have recently identified a mechanism whereby sustained, unremitting overexpression of the genomic organizer SATB1 in tumor microenvironmental DCs drives genome-wide transcriptional programs that, globally, transform DCs into high producers of tumor-promoting IL-6 and immunosuppressive galectin-1 (Tesone, et al., 2016), an inflammatory axis previously shown to ablate anti-tumor immunity and accelerate malignant progression in IL-6 responsive tumors (Rutkowski, et al., 2015). Of note, 22% of transcripts underwent 2-fold changes in expression levels upon *in vivo* silencing of Satb1 specifically in tumor-associated DCs, underscoring the importance of Satb1 as a true genomic organizer and a master regulator of the phenotypic switch of regulatory DCs.

Among the causes for persistent, aberrant overexpression of SATB1 after DC maturation, we found that inflammatory cytokines such as S100A8/A9 are sufficient to up-regulate Satb1 *in vivo*, which dramatically drives the secretion of inflammatory cytokines and chemokines by tDCs (Tesone, et al., 2016). Paradoxically, Satb1 is required for the generation of specific subsets of conventional DCs, but during normal differentiation Satb1 expression needs to be dampened after these cells have acquired MHC-II and therefore immunocompetence. Therefore, *in vivo* silencing of SATB1 specifically in tumor DCs or, more feasibly, neutralization of the immunosuppressive factors produced by SATB1-overexpressing DCs (in particular galectin-1 (Rutkowski, et al., 2015)), emerge as promising immunotherapeutic alternatives.

3.4. DCs are progressively transformed from immunostimulatory to immunosuppressive players through PGE2 and TGF β

Interestingly, the accumulation of regulatory DCs at tumor beds appears to take place at advanced stages of malignant progression. Thus, our studies have evidenced that ovarian cancer-specific T cell responses are initiated by DCs and control tumor growth for relatively long periods (Scarlett, et al., 2012). However, precisely coinciding with the initiation of exponential tumor growth and abrogation of protective immunity, dramatic changes in the phenotype and abundance of DCs take place in the TME. DCs sorted from advanced tumor locations suppress anti-tumor T cells and become accomplices in malignant progression (Scarlett, et al., 2012).

Among the factors typically generated by tumor cells at high levels, PGE2 and TGF- β 1 cooperate to drive the up-regulation of PD-L1 in originally immunocompetent splenic DCs,

and that contributes to their immunosuppressive shift (Scarlett, et al., 2012). Correspondingly, tumor-conditioned media containing high levels of PGE2 and TGF- β 1, transformed DCs from immunostimulatory into immunosuppressive cells that impaired the strong proliferation of tumor-reactive T cells in response to cognate tumor antigen. Importantly, this phenotypic switch required both PGE2 and TGF- β 1 because individual neutralization of either factor in tumor-conditioned media is not sufficient to generate immunosuppressive DCs (Scarlett, et al., 2012). Therefore, tumor-derived factors that include PGE2 and TGF- β 1 also contribute to progressively transform potentially immunocompetent DCs into immunosuppressive cells throughout malignant progression.

3.5. Additional mechanisms of tumor-induced generation of regulatory DCs

An important mechanism of induction of regulatory DCs in the tumor microenvironment is β -catenin/TCF4 signaling, which drives regulatory T-cell responses and, subsequently, tolerogenic responses (Hong, et al., 2015). At tumor beds, DCs metabolize vitamin A and generate retinoic acid, which activates the β -catenin/TCF pathway and promotes Treg accumulation. Interestingly, retinoic acid signaling governs the homeostasis of CD11b⁺ conventional DC subsets in tumor-free hosts, and therefore the immunocompetence of the host (Klebanoff, et al., 2013). The role of retinoic acid in the accumulation and function of the broad range of DC subsets that infiltrate solid tumors therefore deserves further investigation.

Additional mechanisms whereby tumors restrain the immunostimulatory activity of DCs include down-regulation of co-stimulatory CD70 by natural Treg (Dhainaut, et al., 2015); activation of p38 MAPK during DC differentiation (Lu, et al., 2014); and the co-inhibitory signal elicited by CD31 during DC maturation, which decreases NF- κ B nuclear translocation (Clement, et al., 2014). Furthermore, we established that ovarian carcinoma abrogates the expression of the microRNA miR-155 in tDCs as a novel mechanism to control the efficient presentation of tumor-derived antigens to infiltrating T cells (J. R. Cubillos-Ruiz, et al., 2012). Multiple groups subsequently confirmed the critical immunostimulatory role of miR-155 in cancer-associated myeloid cells, including Naldini and colleagues, who demonstrated that myeloid-specific knockdown of miR-155 accelerates tumor growth in a spontaneous model of breast cancer (Zonari, et al., 2013). Finally, emerging studies have associated the expression of PD-1 in ovarian cancer-associated DCs with the impairment of their immunostimulatory function, through a mechanism that results in the inactivation of NF- κ B (Karyampudi, et al., 2016).

Together, multiple lines of evidence demonstrate that as tumors progress, they progressively inhibit the immunocompetence of conventional and inflammatory DCs through multiple complementary mechanisms. At advanced stages of malignant progression, converging tumor-derived signals further transform tumor-associated DCs in situ or arising from distal progenitors into regulatory (immunosuppressive/tolerogenic) cells that contribute to exponential tumor growth by blunting anti-tumor immunity.

4. MECHANISMS OF IMMUNOSUPPRESSION ELICITED BY REGULATORY DCs

Besides preventing effective presentation of tumor antigen-reactive T cells, regulatory DCs inhibit protective T cell-mediated immunity through a variety of direct and indirect mechanisms. Overall, emerging evidence suggests that DCs are as good as eliciting T cell responses as they are at abrogating them. Some important inhibitory mechanisms are as follows:

4.1. Secretion of immunosuppressive cytokines and Treg generation

A major mechanism of immunosuppression elicited by DCs in at least the ovarian cancer microenvironment is mediated by the secretion of galectin-1, a lectin transcriptionally up-regulated in tumor DCs through a Satb1 dependent mechanism (Tesone, et al., 2016). Galectin-1 binds to glycoconjugates that contain N-acetyllactosamine sequences on the cell surface and drives their cross-linking. Due to the dissimilar glycosylation pattern of different T cell subsets, galectin-1 inhibits T cell responses through multiple complementary mechanisms that affect DC immunocompetence (Ilarregui, et al., 2009), cause apoptosis in Th1 and Th17 cells (Toscano, et al., 2007), renders effector T cells unresponsive by cross-linking GM1 ganglioside (Wang, et al., 2009), and promotes the differentiation of tumor-associated Foxp3⁺ Treg cells (Dalotto-Moreno, et al., 2013). Although other cell types secrete galectin-1 in the TME (Rutkowski, et al., 2015), the tolerogenic power of galectin-1 and the relative abundance of regulatory DCs in the microenvironment of multiple tumors indicates that DC-derived galectin-1 is a major activator of cancer immune evasive pathways.

Regulatory DCs can also produce cytokines such as IL-10 or TGF- β that directly inhibit the activity of anti-tumor T cells. In addition, both IL-10 and TGF- β promote the conversion of CD4 T cells into Treg cells, as well as the immunosuppressive activity of natural Treg (Ma, Shurin, Gutkin, & Shurin, 2012; Raker, Domogalla, & Steinbrink, 2015; Torres-Aguilar, et al., 2010).

4.2. Cell contact-dependent mechanisms

In addition to the secretion of immunosuppressive factors, regulatory DCs also blunt T cell responses through the expression of inhibitory ligands on the cell surface. Immune checkpoints comprise multiple regulatory pathways that are crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses. Tumors, however, can co-opt immune-checkpoint pathways as a strategy to abrogate protective T cell-mediated immune responses (Pardoll, 2012). Regulatory DCs in cancer have been shown to express programmed cell death ligand 1 (PD-L1) and PD-L2 which engage their receptor, PD-1, on the T cell surface to transmit inhibitory signals (Shurin, et al., 2013). Supporting initial reports by W. Zou and colleagues (Curiel, et al., 2003), we found that most DCs infiltrating human ovarian tumors expressed significant levels of PD-L1, while T cells in matching specimens expressed the PD-1 receptor (J. R. Cubillos-Ruiz, et al., 2009). Consistent with the perivascular homing of tDCs (Conejo-Garcia, et al., 2005), a barrier of non-tumor PD-L1⁺ cells surrounding tumor islets was found in several human

histological sections. Functionally confirming the immunoregulatory role of PD-L1 on tDCs, we established that selective antibody-mediated blockade of PD-L1 in mouse tDCs was sufficient to induce the activation and proliferation of antigen-specific T cells *in vivo* at tumor locations (J. R. Cubillos-Ruiz, et al., 2009).

Besides PD-1 ligands, our studies further demonstrated that regulatory DCs in the human ovarian cancer microenvironment express butyrophylins that share the CD277 antigen (BTN3A1 and BTN3A3) (J.R. Cubillos-Ruiz, et al., 2010). We found that BTN3A1 is consistently expressed in stromal, as well as tumor cells in the microenvironment of human advanced ovarian carcinoma specimens, both of primary and metastatic origin. Nonetheless, MHC-II+ myeloid antigen-presenting cells, including DCs and macrophages, expressed substantially higher levels of surface BTN3A1 compared to other tumor-infiltrating leukocyte subsets (J.R. Cubillos-Ruiz, et al., 2010). BTN3A1 was rapidly up-regulated in monocyte-derived DCs of cancer-free individuals by multiple inflammatory cytokines and hypoxia-induced mediators commonly found in the tumor microenvironment, including VEGF, and CCL3. Most importantly, however, engagement of BTN3A1 on the surface of TCR-stimulated T cells inhibited their proliferation and production of Th1 cytokines by dampening cFLIP expression (J.R. Cubillos-Ruiz, et al., 2010). There are at least 16 different BTN and BTNL molecules in humans, suggesting that their combined effects could act in concert to drive immune privilege at tumor locations. BTN molecules have therefore emerged as potential negative modulators of anti-tumor immunity.

4.3. Production of catabolic enzymes

Regulatory DCs at tumor beds also drive immunosuppression through the production of catabolic enzymes that deplete Amino Acids that are essential for the effector function of T cells. Among them, the tryptophan catabolic enzymes IDO1 and IDO2, which appear to have redundant and non-redundant activities, are particularly important for maintaining tolerance not only in the TME, but also in multiple inflammatory conditions (Huang, et al., 2013; Metz, et al., 2014; Munn & Mellor, 2016). Besides IDO, regulatory DCs (in addition to macrophages) also produce arginase, which utilizes L-arginine (Norian, et al., 2009; Scarlett, et al., 2012), at least in mouse tumor models. Finally, as their immature myeloid precursors, regulatory DCs can also produce iNOS, responsible for inhibiting immune responses through nitric oxide production (Zhong, et al., 2012).

5. THERAPEUTIC OPPORTUNITIES FOR TARGETING REGULATORY DCS

A better mechanistic understanding of the accumulation of regulatory DCs at tumor beds and their immunosuppressive activities should lead to targeted interventions to boost protective anti-tumor immunity. These interventions could complement other immunotherapies (e.g., T cell adoptive transfer) or exert additional benefit in patients that previously did not respond to checkpoint inhibitor blockade. Several promising approaches have been reported that target tDCs, demonstrating the clinical feasibility of reprogramming tDCs to become immunostimulatory, boost endogenous anti-tumor immunity and reduce tumor progression.

5.1. Promoting the immunostimulatory potential of tumor-associated DCs with nanomaterials

Given their immunosuppressive activity, an obvious approach to boost anti-tumor immunity would be to eliminate regulatory DCs through antibodies or antibody-targeted immunotoxins. Our studies support the feasibility and effectiveness of this approach in preclinical models of ovarian cancer (Huarte, et al., 2008). However, because regulatory DCs appear to retain their phagocytic activity and capture antigens at tumor beds, more effective therapeutic approaches reverse the inhibitory activity of tDCs, restoring their capacity to boost protective immunity *in vivo*, effectively alleviating immune suppression and restoring anti-tumor immunity. This approach is feasible in ovarian cancer, due to the compartmentalized nature of the disease. Thus, complexing biocompatible polymers and bioactive RNA is sufficient to transform DCs in the TME from an immunosuppressive to an immunostimulatory cell type, both *in vivo* and *in situ* in orthotopic tumor models (J. R. Cubillos-Ruiz, et al., 2012; J. R. Cubillos-Ruiz, et al., 2009). The immunogenic boost is sufficient to induce significant survival increases in aggressive tumor models, and involves a combination of TLR activation by both the polymer and the payload, and functional silencing activity of the RNA cargo. Most importantly, fluorescent labeling of untargeted nanomaterials demonstrated that regulatory DCs at ovarian cancer locations were the only cells that readily engulfed the nanoparticles due to their inherent phagocytic abilities (J. R. Cubillos-Ruiz, et al., 2012; J. R. Cubillos-Ruiz, et al., 2009). Taking advantage of the exquisite phagocytic capacity of tDCs, we determined that nanoparticles encapsulating siRNA targeting PD-L1 expression could selectively and persistently silence this immunosuppressive mediator in tDC of mice bearing metastatic ovarian cancer (J. R. Cubillos-Ruiz, et al., 2009). Consistently, the combined action of TLR activation signals and simultaneous PD-L1 downregulation rendered regulatory tDCs highly immunostimulatory, thereby eliciting potent anti-tumor immune responses capable of extending host survival (J. R. Cubillos-Ruiz, et al., 2009). We further exploited this unique nanotechnology approach to selectively restore miR-155 expression in ovarian cancer microenvironmental DCs (J. R. Cubillos-Ruiz, et al., 2012). Strikingly, nanocarrier-directed miR-155 supplementation efficiently altered the global transcriptional profile of tDC *in situ* and concomitantly silenced several immunosuppressive mediators including *Satb1*, SOCS1 and CD200, thereby transforming tDCs into potent local antigen-presenting cells that support anti-tumor T cell function (J. R. Cubillos-Ruiz, et al., 2012). Therapeutic administration of miR-155-loaded nanoparticles that restore tDC function induced significant survival increases in ovarian cancer-bearing mouse models, and tumor rejection was evident in ~30% of animals when nanoparticles were concurrently administered with synergistic CD40 agonists (J. R. Cubillos-Ruiz, et al., 2012). Besides supplementing immunostimulatory miRNAs, another effective way of re-programming the transcriptional profile of ovarian cancer-associated DCs is to deliver functional siRNA oligonucleotides directly silencing *Satb1*, a gene target of miR-155 (Tesone, et al., 2016).

5.2. Pharmacological and biological interventions

Subverting the function of tDCs by mimicking innate signaling pathways has shown promise in pre-clinical models as a therapeutic measure to enhance survival. These strategies leverage the inherent plasticity of DCs by triggering licensing and activation through innate

signaling pathways, resulting in the reinvigoration of anti-tumor immune responses. We have previously shown that the combination of agonistic CD40 and TLR3 ligands activates tDCs in the ovarian tumor microenvironment, resulting in enhanced phagocytic activity, upregulation of costimulatory ligands, and an increase in IL-12 production (Scarlett, et al., 2009). Importantly, this treatment induced enhanced engulfment and processing of tumor-associated antigens and migration of antigen-loaded dendritic cells to tumor draining lymph nodes, where endogenous antigen-specific T cells were activated followed by their infiltration into tumor islets. Thus, synergistic administration of agonistic CD40 and TLR3 ligands significantly increased the survival of mice bearing an aggressive orthotopic model of ovarian cancer (Scarlett, et al., 2009). Additional groups have demonstrated the promise of this synergistic combination using a therapeutic vaccination strategy. Agonistic CD40 ligation and TLR7 ligands significantly enhanced vaccination in a murine model of melanoma (Ahonen, et al., 2008) and have shown promise stimulating naïve human CD8 T cells against the melanoma differentiation antigen MART1 (Klechevsky, et al., 2010).

The intracellular protozoan *Toxoplasma gondii* has a preferential tropism for DCs and macrophages. Taking advantage of the ability of *T. gondii* to invade DCs, elegant studies have utilized an invasive but avirulent strain of *T. gondii* to target tDCs in the ovarian tumor microenvironment in mouse models. Administration of the modified strain of *T. gondii* to mice bearing advanced ovarian cancer induced an expansion of tumor-reactive T cells and conferred significant survival in treated animals (Baird, et al., 2013). Interestingly, the therapeutic benefit conferred by *T. gondii* was abolished in IL-12 deficient animals but was independent of MYD88 signaling (Baird, et al., 2013). Other groups have used attenuated strains of *Listeria monocytogenes*, a normally pathogenic bacterium, to target phagocytic DCs in order to induce potent anti-tumor responses against cancer stem cells (Yang, et al., 2014). In patients with pancreatic ductal adenocarcinoma, treatment with a modified mesothelin-secreting *Listeria monocytogenes* in combination with GM-CSF and low-dose cyclophosphamide induced an increase in overall survival, compared to patients treated with the GM-CSF/cyclophosphamide alone (Le, et al., 2015). Importantly, similar to the other studies, this combination significantly increased the proportions of mesothelin-specific CD8 T cells compared to monotherapy alone (Le, et al., 2015).

Impairment of metabolic pathways have also proven to be effective in reversing the phenotype of regulatory DCs. For example, due to the aforementioned role of retinoic acid in promoting a regulatory phenotype in DCs, pharmacologic inhibition of vitamin A-metabolizing enzymes or the β -catenin/TCF4 pathway has been shown to significantly reduce tumor growth in experimental models, which is associated with a significant reduction in retinoic acid-synthesizing enzymes in tumor-draining lymph nodes (Suryawanshi & Manicassamy, 2015). In addition, given that IRE1 α -XBP1 signaling sustains both tDC dysfunction (J. R. Cubillos-Ruiz, et al., 2015) and cancer cell-intrinsic growth and metastasis (Chen, et al., 2014), there is significant interest in developing targeted therapies against this conserved branch of the ER stress response. While it is technically challenging to develop direct XBP1 inhibitors, the formation of the active (spliced) *Xbp1* form can be readily targeted via its dependency on the IRE1 α endoribonuclease domain. Indeed, this dual enzyme is suitable to small molecule targeting, and multiple inhibitor classes have been identified from various independent small molecule screens (Tang, et al.,

2014). Therefore, we are currently testing whether such IRE1 α -XBP1 inhibitory compounds could be exploited as a new therapeutic strategy that restrain malignant cell survival while eliciting enhanced anti-tumor immunity via tDC re-programing.

6. CONCLUDING REMARKS

Therapeutic blockade of immune checkpoints such as CTLA4 or PD1 has shown significant promise by inducing clinical responses in some types of cancer. However, these treatments are often linked with severe “off target” toxicities that impair the patient’s ability to effectively respond to therapy. Because DCs are at the nexus of inducing potent CD8 T cell-mediated responses, a more targeted approach to eliciting potent anti-tumor immune responses is to enhance the immunostimulatory potential of tDCs within the TME. Thus, although rare populations of DCs with strong antigen-presenting activity have been identified in cancer (Broz, et al., 2014), DCs with immunosuppressive, rather than immunostimulatory activity, are a more common occurrence in many advanced solid tumors of different histological origins. Although their frequency varies between different tumors and in different patients, solid ovarian tumor masses (unlike ovarian cancer ascites) appear to contain particularly high numbers of inflammatory, regulatory DCs. How infiltration of regulatory DCs correlates with the frequencies and activity of effector lymphocytes, how they interact with potentially immunostimulatory DCs, and how they influence the patient’s outcome, remain important questions that demand mechanistic answers.

Several studies have demonstrated that reprogramming tDCs is a safe and effective strategy to induce antigenic spreading and long-lasting protective anti-tumor immune responses. These strategies are based upon years of work aimed at understanding the ontogeny and function of DCs in various experimental and pathological conditions. As our understanding of the transcriptional, metabolic, and epigenetic mechanisms driving regulatory DC function increases, the likelihood of developing targeted, potent, and even tumor-specific DC-based immunotherapies is promising.

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Abbreviations

DC	Dendritic Cell
ER	Endoplasmic Reticulum
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
iNOS	inducible nitric oxide synthase
IRE1α	Inositol-requiring enzyme 1 alpha

MAPK	Mitogen-activated protein kinase
PD1	Programmed death 1
PD-L1	Programmed death-ligand 1
PEI	Polyethylenimine
PERK	PRKR-like endoplasmic reticulum kinase
PGE2	Prostaglandin E 2
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
siRNA	small interfering RNA
tDC	Tumor-associated DC
TGFβ	Transforming Growth Factor beta
TLR	Toll-like receptor
TME	Tumor Microenvironment
VEGF-A	Vascular Endothelial Growth Factor
UPR	Unfolded Protein Response
XBP1	X-box binding protein 1

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Figure 1. Pathological myelopoiesis, ER stress and Satb1 overexpression converge to transform DCs from an immunostimulatory to an immunosuppressive, tumor-promoting cell type

During emergency myelopoiesis, myeloid differentiation is dysregulated by tumor-promoting factors such as S100A8/A9, IL-6 and VEGF-A, resulting in the expansion of immature myeloid subsets and their mobilization to lymphatic locations and tumor beds. Pathological myelopoiesis involves disruption in the differentiation program of common myeloid progenitors (CMP) into granulocyte-macrophage progenitors (GMP), which give rise to PMN-MDSCs. Differentiation of macrophages and DCs is also subverted, resulting in the generation of monocytic MDSC (M-MDSC), which turn into macrophages and DCs in the TME. DC maturation is impaired by tumor-derived factors, including VEGF-A, promoting anergy and ineffective priming of T cells and, eventually T cell exhaustion. Inflammatory DCs originating from both monocytes and true DC precursors due to inflammatory stimuli undergo oxidative stress at tumor beds. Generation of reactive oxygen species (ROS) in inflammatory DCs results in ER stress and subsequent upregulation of XBP1, inducing inflammatory DCs to abnormally accumulate lipids, resulting in an inhibitory phenotype and diminished ability to induce robust anti-tumor T cell responses. In addition, inflammatory DCs acquire profound suppressive activity in the TME under the influence of tumor-derived PGE2 and TGFβ. Furthermore, tumor- and myeloid cell-derived S100A8/9 drives unremitting expression of the genomic organizer SatB1 in pre-DC-derived inflammatory DCs, driving hyper-secretion of IL-6 and galectin-1, which has a strong direct immunosuppressive activity on T cells. Plasmacytoid DCs (pDCs) also drive Treg differentiation at tumor beds, further contributing to the suppression of anti-tumor immunity. The immunosuppressive activity of tDCs derived from M-MDSCs remains to be determined.

Figure 2. Summary of potential therapeutic strategies to convert regulatory DCs into immunostimulatory DCs *in vivo*

References from published experimental models are included.