DENDRITIC CELL CONTROL OF IMMUNE RESPONSES

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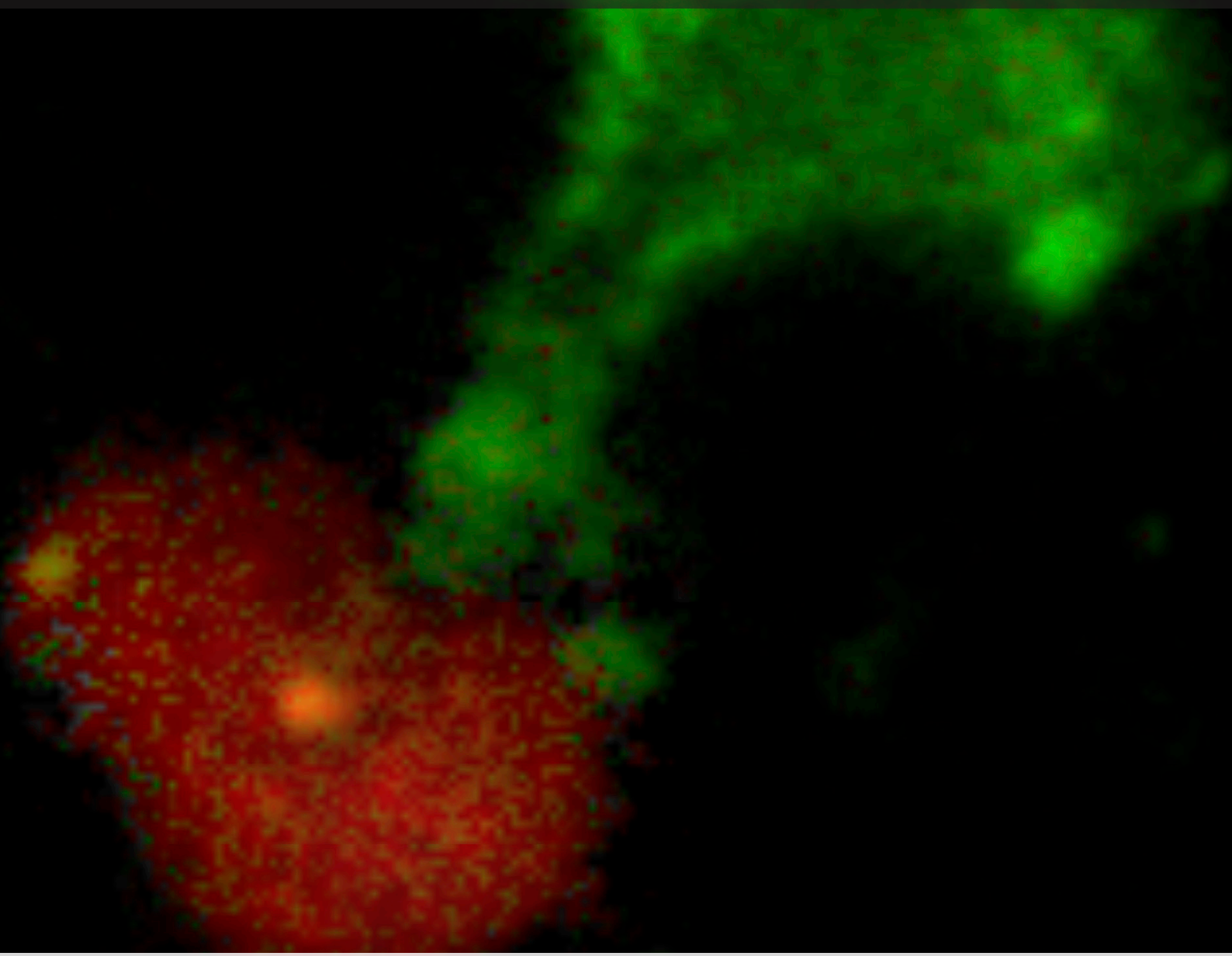

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DENDRITIC CELL CONTROL OF IMMUNE RESPONSES

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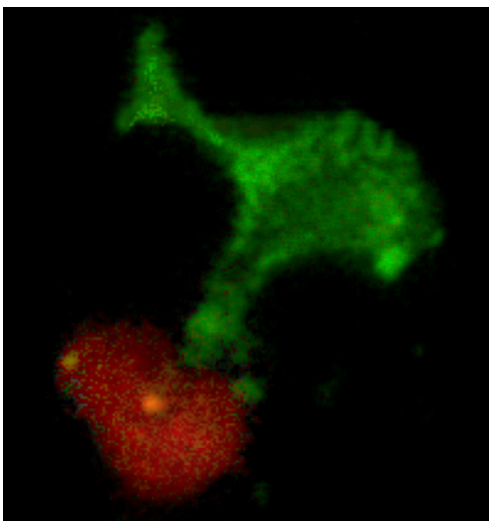

Two photon image of a dendritic cell (green) interacting with a specific T cell (red) in the pancreatic lymph node of an NOD mouse.

Image by Penelope Anne Morel

Dendritic cells (DC) are among the first cells to encounter pathogens and damage in peripheral tissues and, upon activation, DC migrate to lymph nodes where they activate and educate T cells to initiate and shape the immune response. DC present pathogen-derived antigen to T cells and drive T cell differentiation into particular effector cells through the expression and secretion of co-stimulatory molecules and cytokines respectively. The study of DC biology has included the identification of multiple DC subsets in tissues and lymphoid organs, the differentiation and plasticity of DC subsets, the functional consequences of DC interaction with pathogen, control of DC migratory properties and the impact of DC on T cell activation and differentiation. In recent years sophisticated systems biology approaches have been developed to deepen our understanding of DC function. These studies have identified differences between DC subsets located in various tissues and critical factors that drive the outcome of the interaction between DC and T cells. DC are currently being used in in various clinical therapeutic settings, including as vaccines for cancer and autoimmune disease. A clear understanding of DC factors that contribute to specific immune responses is vital to the success of DC based therapies. This research topic will give a comprehensive overview of current issues in DC biology and provides an update on the clinical uses of DC in the therapy of autoimmunity and cancer.

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Dendritic cell control of immune responses

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Keywords: dendritic cells, immuno response, autoimmune diseases, inflammation, cancer vaccines

Dendritic cells (DC) play a critical role in the initiation of the immune response, acting as sentinels in the tissues, reacting to invading pathogens and then inducing the activation and differentiation of naïve T cells. Since their discovery by Ralph Steinman in the 1970s, we have learned a great deal about DC biology and function. Distinct DC subsets exist in specific tissue niches and within the secondary lymph nodes, and the study of the phenotype and function of DC subsets in mice and humans has been an area of great interest. DC influence the immune response by directing the differentiation of T cells into functional subtypes important for elimination of the relevant pathogen. DC also contribute to disease pathogenesis, such as autoimmunity and cancer, through failures in self-tolerance and promotion of an immunosuppressive environment, respectively. In addition, because of their role in molding T cell responses, DC have been tested in therapeutic settings in both autoimmunity and cancer. In this research topic, 11 articles cover many aspects of current DC biology, ranging from the classification and function of DC subsets, roles of DC in disease pathogenesis to current use of DC in the therapeutic setting.

The topic begins with two reviews of normal DC function; one is focused on the important topic of cross-presentation (1) and the other on human plasmacytoid (p)DC (2). Cross-presentation is the means by which DC are uniquely able to take up, process, and present exogenous antigens in MHC class I molecules and this review (1) describes novel findings on the role of intracellular vesicular traffic in this process and how it is influenced by inflammatory signals. pDCs were first identified as type 1 interferon-producing cells following acute viral infection and have also been shown to have tolerogenic properties. The review by Mathan et al. (2) focuses on interactions between human pDCs and other cells of the immune system with a particular emphasis on cell surface proteins that facilitate these interactions.

The next four articles in this research topic are original research focusing on various aspects of DC function *in vivo* and *in vitro* (3–6). DC maturation is induced by interaction with pathogen-derived molecules such as LPS, but infections also induce production of a large number of cytokines. Work by Hartmann et al. (3) examined the effects of virally induced cytokines on the maturation and function of human DC. These studies identified a set of five cytokines that are critical for the induction DC maturation. In addition, this study emphasizes a systems approach to studying the complex effects of cytokine-induced DC maturation (3). DC play an important role in maintaining tolerance to self-antigens and the second article in this section describes the role of early IL-10 production in induction of tolerance to a self-antigen (6). This article builds on the intriguing observation by the same group that immunization with foreign antigen induces a rapid upregulation of pancreatic enzymes in splenic DC that is correlated with the induction of immunity. The present article shows that immunization with a self-antigen fails to induce pancreatic enzymes and demonstrates a role for IL-10 in this phenomenon (6). Work by Kokulus et al. (5) demonstrates the influence of mild chronic cold stress on the phenotype and function of DC in normal and tumor-bearing mice. These studies highlight the importance of regulating ambient temperature when conducting experiments with experimental animals and the impact of non-physiologic temperature. Chronic human inflammatory diseases are often characterized by changes in circulating monocyte and DC populations. The final article in this section describes a novel flow cytometry panel, using CD4 as a lineage marker that allows the enumeration of monocyte, DC, and lymphocyte populations in a single panel (4). This panel was validated in patients with immunodeficiency, cancer, and inflammatory conditions.

The final group of five reviews highlights the role of DC in either causing or ameliorating disease (7–11). Autoimmune diseases are characterized by a breakdown of self-tolerance followed by an immune response that causes damage to normal tissues. DC play roles at all stages of the autoimmune response and these are outlined in a review that focuses specifically on type 1 diabetes (T1D) (11). This review also highlights the potential for using DC to prevent or treat T1D and discusses the latest clinical trials using or targeting DC in this disease (11). The second review focuses on the many types of immunoregulatory DC and their role in preventing inflammatory conditions such as autoimmunity, transplant rejection, and atopic diseases (9). The last three reviews in this research topic focus on the therapeutic use of DC as cancer vaccines. In the first of these reviews, the importance of tertiary lymphoid structures in the development of effective anti-tumor immunity is discussed (8). The authors show that injection of DC, expressing the transcription factor Tbet, into the tumor stimulates the generation of tertiary lymphoid structures and contributes to tumor eradication. The second provides an overview of the preparation, functional characteristics, and use of human DC in cancer vaccines, with particular emphasis on the culture methods, maturation cocktails, antigen formulation, and routes of delivery that are currently in use (7). The final review in this research topic

focuses on the barriers to the generation of effective DC vaccines in cancer patients (10). In particular, this review outlines the challenges posed by immunosuppressive monocyte populations, prevalent in cancer patients, which impede the generation of immunostimulatory DC vaccines.

Thus, this research topic provides a timely overview of some of the recent advances in DC biology and we look forward to many new developments in this exciting field.

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An updated view of the intracellular mechanisms regulating cross-presentation

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Cross-presentation involves the presentation of peptides derived from internalized cargo on major histocompatibility complex class I molecules by dendritic cells, a process critical for tolerance and immunity. Detailed studies of the pathways mediating cross-presentation have revealed that this process takes place in a specialized subcellular compartment with a unique set of proteins. In this review, we focus on the recently appreciated role for intracellular vesicular traffic, which serves to equip compartments such as endosomes and phagosomes with the necessary apparatus for conducting the various steps of cross-presentation. We also consider how these pathways may integrate with inflammatory signals particularly from pattern recognition receptors that detect the presence of microbial components during infection. We discuss the consequences of such signals on initiating cross-presentation to stimulate adaptive CD8 T cell responses.

Keywords: cross-presentation, major histocompatibility complex class I, pattern recognition receptor, Toll-like receptor, vesicular traffic, phagosomes, endosomes, dendritic cells

INTRODUCTION

Classically, endogenous antigens such as proteins synthesized by virally infected cells or tumor cells, are presented on major histocompatibility class I (MHC I) molecules for detection by CD8 T cells. However, a seminal study by Bevan (1) showed that in animals immunized with fully allogeneic cells, cytotoxic CD8 T cell responses were seen specific for minor antigens from the graft that were presented on MHC I molecules of the host (1). This finding indicated that antigens from the transplanted cells could be internalized by antigen presenting cells (APC) of the host and presented on the host MHC I molecules. Bevan termed the activation of CD8 T cells to this process of antigen transfer as "cross-priming," and later the actual process of antigen transfer was called "cross-presentation" (2). A once poorly defined phenomenon, cross-presentation is now considered to be a critical mechanism to mediate immune responses against infectious agents and tumors as well as to induce peripheral tolerance (3).

The importance of cross-presentation becomes apparent given the existence of several viruses that exhibit strict tissue tropisms such as papilloma virus where infection is mainly confined to epithelial cells in the skin barrier (4). Other examples for viruses that do not infect APC include encephalomyocarditis virus (EMCV) and semliki forest virus (SFV) (5). Additionally, some viruses such as herpes simplex virus (HSV), measles, retrovirus, canarypox virus, vaccinia virus, and lymphocytic choriomeningitis virus infect APC, but impair direct presentation of antigen (6–13). Additionally, cross-presentation has been demonstrated to play a critical role in mediating CD8 T cell immune responses against parasitic infections such as *Toxoplasma gondii* (14). Cross-priming has also been studied in the context of bacterial infections such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*. In these infections, host defense is primarily mediated by dendritic cells (DC) that phagocytose infected apoptotic cells and mediate cross-priming, thus allowing for effective cytotoxic T lymphocytes (CTL) responses against the pathogens (15–17). Hence, cross-presentation allows for a mechanism through which the antigen can be presented by the APC without the need for direct infection.

Although other phagocytes have been reported to cross-present antigen, DC are considered to be the primary cross-presenting cell. The superior ability of DC to cross-present is largely attributed to their antigen processing capacity. Endocytic pathways in DC preserve and retain antigen epitopes via low lysosomal proteolysis and expression of protease inhibitors (18). This aspect makes sense when one considers that DC pick up antigen in the peripheral tissue and migrate for several hours-days (12–24 h for dermal DC and 3 days for Langerhans cells) reaching the lymph nodes (19). Thus, instead of being processed and degraded prematurely, retention of antigen would allow optimal cross-presentation for subsequent recognition by lymph node resident naïve CD8 T cells. However, DC subsets are heterogeneous in their ability to cross-present antigens. Subsets such as conventional splenic and lymph node resident CD8α^+ DC, migratory DC populations such as lung and dermal CD103$^+$ DC as well as monocyte-derived inflammatory DC excel at cross-presentation (20–24). It is still unclear why these DC subsets are specialized for cross-presentation. Interestingly, conventional CD8α^+ DC as well migratory CD103$^+$ DC populations appear to rely exclusively on Batf3 and IRF8 transcription factors for their development (25–28). Therefore, it is curious to ask if the ability to cross-present is developmentally controlled by

the Batf3/IRF8 transcription programs that enable these subsets to have unique and specialized compartments geared toward cross-presentation. On the other hand, *in vitro* studies argue that the ability of splenic CD8α$^+$ DC to cross-present antigen is induced as a subsequent step in maturation aided by cytokines such as granulocyte-macrophage colony-stimulating factor (GMCSF) or exposure to microbial products (29).

Recent studies in human DC have proposed the lymphoid and non-lymphoid resident BDCA3$^+$ (CD141$^+$) DC to be the human counterparts of the cross-presenting murine lymphoid CD8α$^+$ DC and non-lymphoid CD103$^+$ DC (30–34). The BDCA3$^+$ DC subset is indeed an attractive candidate for the human homolog as it shares with the murine CD8α$^+$ and CD103$^+$ DC several cell surface markers, including DNGR1 and XCR1, transcription factors such as Batf3 and IRF8, along with excelling at *in vitro* cross-presentation assays. However, evidence also exists contradicting the superiority of the BDCA3$^+$ DC subset at cross-presentation (35–37). Furthermore, patients harboring an autosomal dominant mutation in *IRF8* selectively lose BDCA-1$^+$ DCs but not BDCA3$^+$ DCs in the peripheral blood, indicating that BDCA3$^+$ DC is at least not developmentally regulated in the same manner as murine cross-presenting subsets (38). Additionally although Batf3 deficiency impairs development of BDCA3$^+$ DC *in vitro*, humanized mice reconstituted with Batf3 deficient progenitors still display sufficient and comparable numbers of BDCA3$^+$ DCs (39). Thus, even though the human BDCA3$^+$ DC subset appears to be functionally related to the murine CD8α$^+$ and CD103$^+$ DC, further studies are warranted to determine their developmental program and subsequent specialization for cross-presentation.

Several groups have also focused their efforts on revealing the intracellular pathways and molecular mechanisms mediating cross-presentation at steady state. Three major mechanisms have been determined. The phagosome-to-cytosol pathway involves escape of the antigen into the cytosol, possibly mediated by Sec61, followed by degradation by the proteasome and transport of peptides into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP) to be loaded onto MHC I molecules (40, 41). The ER-phagosome fusion pathway involves fusion of the ER-Golgi intermediate compartment (ERGIC) with the phagosome, leading to the formation of a so called "ERgosome," where ERGIC components such as TAP and components of the peptide loading complex are recruited directly to the phagosome. This recruitment is mediated through pairing of the ER SNARE Sec22b with the plasma membrane SNARE syntaxin-4, which is also found on phagosomes (42). In this model, the antigen still requires escape into the cytosol for proteasomal degradation and is then imported back to the "ERgosome" by TAP to be loaded onto MHC I molecules (43, 44). In contrast to the cytosolic pathways, the vacuolar pathway involves direct processing of the antigen within the phagosome by endocytic proteases such as cathepsins and subsequent loading of peptides onto MHC I molecules (45).

Some of the above pathways suggest that cross-presentation takes place in a specialized intracellular compartment. This compartment could be endosomes or phagosomes depending on the size of the exogenous antigen and mode of internalization. For instance, in the ERgosome model, cross-presentation takes place in a subcellular compartment bearing characteristics of both the ERGIC and the endosome/phagosome. In this review, we elaborate on the vesicular pathways that serve to bring various components of the cross-presentation machinery to such specialized intracellular compartments. We discuss the unique combination of proteins in these compartments that make it attractive for cross-presentation at steady state and how this compartment might be modified and further optimized for efficient cross-presentation in the scenario of infection. We also review evidence for regulation of cross-presentation during microbial stimulation and discuss if this process can still take place at steady state.

NATURE OF THE CROSS-PRESENTING COMPARTMENT AT STEADY STATE

In recent years, several groups have elucidated the architecture of the "cross-presenting" compartment at steady state. This compartment contains several identified proteins brought in by distinct vesicular pathways. These proteins may all be present in the compartment. Alternatively, these vesicular pathways may be mutually exclusive of one another, culminating in the presence of only some of these identified proteins. Regardless, these proteins serve as important players in executing different steps of the cross-presentation response (**Figure 1**).

MAINTENANCE OF OPTIMAL ALKALINE pH

As antigens are internalized by endocytosis or phagocytosis, they undergo gradual proteolytic degradation along their journey from early endosomes and phagosomes to lysosomes (**Figure 1A**). Once in lysosomes, antigens are degraded by lysosomal proteases, which could destroy potential peptide epitopes crucial for T cell activation. DC circumvent this problem by expressing low levels of lysosomal proteases (18). Additionally, since most of these proteases function optimally at acidic pH (46), maintenance of a strongly alkaline pH in the cross-presentation compartment would inhibit protease activity, thus preventing overt and premature degradation of antigens. To this end, DC were reported to have high phagosomal pH, reaching values of 7.5–8 in contrast to macrophages which rapidly acidified their phagosomes, reaching values of 4.5–5 following phagocytosis of inert latex beads (47). Alkalinization of phagosomes in DC was attributed to selective recruitment, assembly and functioning of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase NOX2. Finally, NOX2 activity was shown to be crucial for cross-presentation as genetic deletion of NOX2 subunit gp91phox led to abrogation of cross-presentation.

How does NOX2 reach the cross-presenting compartment? Recruitment of NOX2 is facilitated by Rab27a (48), a small guanosine tri-phosphatase (GTPase), which was initially characterized to mediate regulatory exocytosis of secretory vesicles in hematopoietic and non-hematopoietic cells (49). In a separate study, the Rac2 GTPase was demonstrated to also control the recruitment and assembly of phagosomal NOX2 in splenic CD8α$^+$ DC as well as in *in vitro* derived bone-marrow derived DC (50). Additionally, VAMP-8, a SNARE protein which interacts with plasma membrane and phagosomal SNAREs syntaxin-4 and SNAP-23 (51), has also been recently reported to play a role in NOX2 recruitment and in mediating cross-presentation of phagocytic antigen (52). Interestingly, the protozoan *Leishmania* specifically cleaves VAMP-8

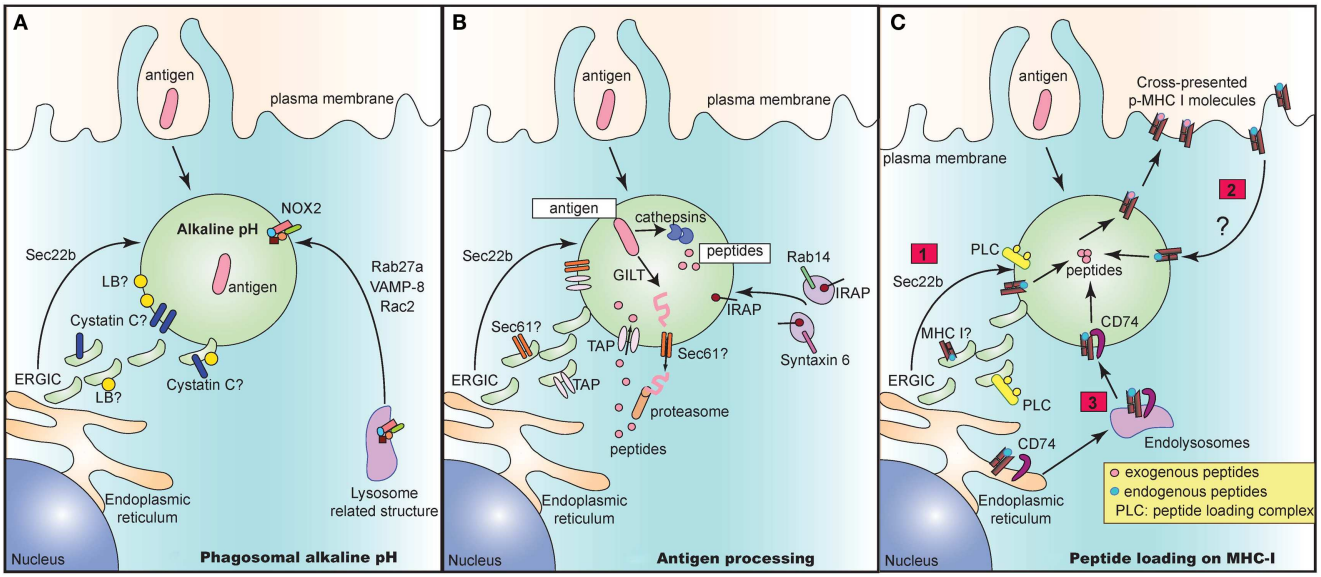

FIGURE 1 | The cross-presentation compartment at steady state. Several vesicular pathways have been proposed to mediate delivery of key proteins that play an integral role in the crucial steps of cross-presentation including maintenance of alkaline pH **(A)**, processing of antigen to peptides **(B)** and subsequent loading of peptides onto MHC I molecules **(C)**. **(A)** Delivery of NOX2 via Rab27a, VAMP-8, and Rac2 is critical for maintaining an alkaline pH in intracellular cross-presenting compartments. Alternatively, recruitment of ERGIC via Sec22b also regulates pH and proteolytic activity in phagosomes. The ERGIC may contain protease inhibitors such as Cystatin C and lipid bodies (LB) that could directly alter phagosomal pH and enzymatic activity of proteases. **(B)** In the vacuolar pathway, antigen is directly processed into peptides by phagosome resident proteases such as cathepsins. In the cytosolic pathway, the antigen may need to be first unfolded by GILT prior to exit into the cytosol via a channel. This mystery translocon may be Sec61 and could be present in ERGIC, thereby recruited to the cross-presenting compartment via Sec22b. Once in the cytosol, antigen is then degraded into peptides and shuttled back into the phagosome via phagosomal TAP. TAP is dependent on Sec22b for its recruitment from ERGIC to endocytic compartments. Finally, IRAP, which is present on Rab14$^+$ and Syntaxin 6$^+$ endosomes, is recruited to phagosomes and mediates trimming of imported peptides that are further optimized for cross-presentation. **(C)** Lastly, processed and trimmed peptides have to now be loaded onto MHC I molecules. Source of MHC I in such compartments is unclear and could either be recruited from (1) ERGIC via Sec22b or (2) recycling from plasma membrane or (3) recruitment from endolysosomal compartments via CD74. In all cases, the peptide loading complex (PLC) is recruited from ERGIC via Sec22b to the compartment and can chaperone loading of exogenous foreign peptides to create "cross-presentable" peptide-MHC complexes.

in phagocytes to prevent NOX2 assembly, thereby acidifying the phagosomes, in order to evade the cross-presentation response. However, it is still unclear if these GTPases and SNARE proteins act in concert or independently of one another to mediate recruitment of NOX2 and in turn to control cross-presentation. Given that VAMP-8 also participates in trafficking of secretory vesicles (53, 54), it is tempting to speculate that VAMP-8 and Rab27a might be present in similar secretory granules and are routed to the cross-presenting compartment upon entry of antigen.

In a second pathway, Sec22b mediated recruitment of ERGIC components has additionally been implicated in the maintenance of an alkaline pH (42). Sec22b silenced DC phagosomes have higher levels of mature cathepsin D, increased proteolytic activity, leading to accelerated degradation of antigen. These results therefore suggest that the ERGIC contains protease inhibitors. Which protease inhibitors could be involved? The cystatin family of protease inhibitors has been implicated to play a role in antigen presentation. Cystatin C was demonstrated to inhibit degradation of CD74, leading to enhanced accumulation of MHC II in endolysosomal compartments (31). Interestingly, cystatin C is abundantly expressed by CD8α^+ DC compared to CD8α^- DC from the spleen (32), and only partially colocalizes with endolysosomal compartments (31, 32). Given that the cellular localization of cystatin C as well as its role in cross-presentation is still unclear, a feasible possibility is that cystatin C could perhaps colocalize with ERGIC and play a role in cross-presentation.

Another explanation for why recruitment of ERGIC would delay phagosome maturation is that the ERGIC may contain lipid bodies (LB) that have been implicated in regulating phagosomal alkalinization and antigen cross-presentation (55). These LB accumulate in the cytosol and on DC phagosomes in an interferon (IFN)-inducible ER-resident GTPase (Igtp) dependent manner. Specifically, Igtp was shown to interact with LB resident adipose differentiation related protein (ADFP) to mediate formation of LB, which were crucial for cross-presentation (55).

ANTIGEN PROCESSING

The cytosolic pathway model of cross-presentation stipulates that once antigen is internalized, it has to make its way out of the endosome/phagosome and into the cytosol for proteasomal degradation (**Figure 1B**). It is generally thought that prior to export into the cytosol, antigens may need to be unfolded. For certain antigens, this is a challenge owing to specific di-sulfide bonds holding the structure of the antigen together. In this case, gamma-interferon-inducible lysosomal thiolreductase (GILT) has been shown to be critical for cross-presentation of di-sulfide bonds containing antigen derived from HSV infected cells (56). Once unfolded, antigen is then routed to the cytosol through a channel,

the identity of which still remains enigmatic and controversial. Sec61, a translocon involved in the ER associated degradation pathway (ERAD) was regarded as a top candidate given that blocking Sec61 activity by using *Pseudomonas aeruginosa* bacteria exotoxin A resulted in loss of cross-presentation of soluble OVA antigen (41). However, the evidence for exotoxin A directly and solely blocking Sec61 channel activity is still lacking. Interestingly, DC lacking Sec22b SNARE protein via short hairpin ribonucleic acid (shRNA) targeted deletion, showed impaired antigen export from endocytic compartments, thus arguing for the recruitment of an ERGIC resident translocon channel (42). Further studies analyzing phagosomal proteomics of these Sec22b sufficient and deficient DC would be integral to revealing the identity of the enigmatic translocon.

Once in the cytosol, it is well accepted that the antigen undergoes proteasomal degradation resulting in the generation of peptides. These peptides are then reimported by TAP into the lumen of the cross-presenting compartment (57). TAP is an ER and ERGIC resident protein that has been demonstrated to be recruited to phagosomes in a Sec22b dependent manner (42). Several groups have confirmed the dependence of cross-presentation on TAP, although in the case of certain bacterial antigens, cross-presentation can take place even in the absence of TAP via the vacuolar pathway where antigens are processed within endosomes and phagosomes by resident proteases (3).

Upon internalization of exogenous antigens, newly generated peptides can be trimmed by endosomal insulin-regulated aminopeptidase (IRAP) to be further optimized for cross-presentation (58). $IRAP^{-/-}$ DC are impaired in their ability to cross-present soluble and particulate antigen, thus implicating the importance of IRAP in cross-presentation. IRAP colocalizes with $Rab14^{+}$ and syntaxin 6^{+} endosomes at steady state, and is recruited to phagosomes after antigen uptake (58, 59). Whether IRAP depends on Rab14 GTPase and syntaxin 6 SNARE proteins for its delivery to the phagosomes remains to be studied.

PEPTIDE LOADING ON MHC I

Processed and trimmed peptides are now faced with the possibility of being loaded on MHC I molecules that are present within endosomes and phagosomes (**Figure 1C**). A question that remains is where these MHC I molecules are recruited from. An attractive possibility is that MHC I molecules are present in the ERGIC and that perhaps Sec22b can deliver MHC I along with its chaperone proteins such as calreticulin and tapasin to the cross-presenting compartment. However, analysis of MHC I molecules in cell lines has revealed that MHC I molecules are transiently trafficked through the ERGIC at steady state. In fact, MHC I accumulated in ERGIC only in conditions where these molecules are not bound to high affinity peptides which could occur in the absence of TAP or calreticulin or when traffic out of the ERGIC is blocked (60–64). Whether MHC I trafficking in APC occurs similarly is still unclear.

An alternative possibility is that MHC I may be derived from the plasma membrane. Indeed, endocytosis and subsequent recycling of MHC I has been extensively documented in several cell lines where internalized MHC I are delivered to endosomal recycling compartments (ERC) in a step prior to being re-routed to the plasma membrane (63). Trafficking patterns of MHC I in APC are less clear. Some studies in APC confirm the reliance on plasma membrane derived MHC I, where internalization of surface MHC I molecules was shown to be dependent on a conserved tyrosine within the cytosolic domain of the MHC I, and to a lesser extent on a conserved serine phosphorylation site (65, 66). Mutations in these conserved sites resulted in inhibition of cross-presentation both *in vitro* and *in vivo*. However, given the strong evidence in cell lines for accumulation of MHC I in ERC, further studies are warranted to determine the contribution of MHC I molecules recycling through the ERC to cross-presentation.

Finally, it was also recently shown that CD74, which was originally characterized to route MHC II molecules from the ER to lysosomal compartments (67), was also important in mediating cross-presentation of viral and cell-associated antigen (68). CD74 was found in complex with immature endoglycosidase sensitive MHC I, indicating that it associates with newly synthesized MHC I in the ER (69). In $CD74^{-/-}$ DC, MHC I molecules were present to a lesser extent in LAMP-1^{+} compartments, implying that CD74 delivers MHC I from the ER to endolysosomal compartments.

NATURE OF THE CROSS-PRESENTING COMPARTMENT DURING INFECTION

In spite of these studies detailing the molecular and cellular makeup of cross-presenting compartments at steady state, the mechanisms underlying regulation and remodeling of this compartment during infection remain largely undefined. Upon uptake of microbial or infected cellular cargo, the phagosomal or endosomal compartment can be substantially modified by the acquisition of pattern recognition receptors (PRRs) (16). PRRs are evolutionarily conserved receptors that recognize and respond to conserved pathogen-associated molecular patterns (PAMPs) which are unique to microbes (70). Upon PRR engagement, intracellular signal transduction pathways are initiated such as those mediated by nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs), and IFN-regulatory factors (IRFs) (71). These pathways are critical for providing immunity against several pathogen infections.

Several families of mammalian PRRs have been identified, namely Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like (NOD-like) receptors (NLRs), RIG-I like receptors (RLRs), and C-type lectin receptors (CLRs) (72). Indeed, TLRs were the first family of PRRs to be studied in detail (73–75). We focus here on recent developments in the biology of TLR that enable the recruitment of these receptors and assembly of their signaling machinery. This recruitment aids in the transduction of responses from the compartment itself, allowing localized phagosomal or endosomal specific control of responses, including presentation of exogenous antigen on MHC I and MHC II molecules. To signal and regulate presentation of exogenous antigen from endosomal compartments, TLRs first need to be recruited to the relevant compartment.

TLR4

TLR4 is a plasma membrane resident "surface" TLR that can be endocytosed and also signal from intracellular compartments upon interaction with its ligand lipopolysaccharide (LPS). TLR4 initially engages toll-interleukin 1 receptor (TIR)

domain-containing adaptor protein (TIRAP) and myeloid differentiation primary-response protein (MyD88) to initiate signal transduction from the plasma membrane (76). Subsequently, TLR4 is internalized into endocytic compartments and engages TRIF-related adaptor molecule (TRAM) and TIR-domain-containing adaptor inducing IFN-β (TRIF) (76). In fact, blocking TLR4 endocytosis using dynamin inhibitors selectively inhibits TRIF-TRAM mediated IRF3 dependent type I IFN responses without affecting TIRAP-MyD88 dependent signaling (77).

Recently, CD14 was shown to regulate the endocytosis of TLR4 from the plasma membrane during stimulation with LPS (78). TLR4 acts as a cargo for CD14, which transports the receptor and LPS to endosomes in a Syk and PLCγ2 dependent process where TRIF signaling leads to IFN-β production. While CD14 is critical for TLR4 endocytosis and IFN-β production in response to soluble LPS, TLR4 endocytosis in DC can proceed in the absence of CD14 during phagocytosis of *E. coli* or LPS-coated beads, although a lower percent of TLR4 is internalized compared to soluble LPS. Notably, despite lower percent TLR4 internalization in response to these particles, IFN-β production, which relies on TLR4 signaling from endocytic compartments, was unaffected in the absence of CD14. This result suggests that in the case of phagocytosed cargo, TLR4 can accumulate on phagosomes from another source independently of the plasma membrane.

Indeed, the small GTPase Rab11a was shown to play a crucial role in trafficking TLR4 from ERC to phagosomes containing gram-negative and not gram-positive bacteria, leading to IRF3 activation and IFN-β transcription (79). Additionally, adaptor protein 3 (AP-3) also plays a role in recruiting TLR4 and MyD88 from intracellular stores to phagosomes containing TLR4 ligands (80). Interestingly, AP-3 dependent recruitment of TLR4 and MyD88 was crucial for mediating production of pro-inflammatory cytokines selectively in response to phagocytic cargo and not to soluble LPS. Hence, the mode of uptake can dictate the pathway of delivery of TLR4.

It is curious to ask if the recruitment of TLR4 via these regulatory transport proteins contributes toward antigen presentation and adaptive immunity. For MHC II presentation, phagosome autonomous TLR4 signaling led to accelerated phagosome maturation and subsequent degradation of CD74 specifically in phagosomes bearing TLR ligands and not other phagosomes in the same DC (81, 82). Additionally, impaired recruitment of TLR4 to phagosomes in AP-3 deficient mice also led to decreased MHC II presentation (80). However, whether any of these proteins regulate cross-presentation of antigen internalized via endocytosis or phagocytosis remains to be investigated.

TLR9

TLR9 is an endosomal receptor and begins its journey in the ER, where it associates with the chaperone protein Unc-93 homolog B (UNC93B), which mediates its transport to endosomes (83). Recently, recruitment of TLR9 and UNC93B was demonstrated selectively to phagosomes that contained DNA and anti-DNA immunoglobulin (Ig) complexes (84). Importantly, phagosomal TLR9 recruitment did not depend on its ability to sense the presence of TLR9 ligand DNA but instead relied on Fc receptor γ (FcRγ) mediated engagement by Ig complexes. These data suggest that Ig mediated FcRγ signaling leads to recruitment of TLR9 to phagosomes. When these phagosomes also contain the TLR9 ligand DNA, TLR9 signaling is engaged, resulting in IFN-α secretion. Given that engagement of FcRγ prepares phagosomes for optimal TLR9 signaling, it is tempting to speculate that this synergy between FcRγ and TLR9 may also impact subsequent cross-presentation responses of antigen complexed with DNA-Ig aggregates.

TLR2

TLR2 is a cell surface TLR that synergizes with other surface TLRs such as TLR1 and TLR6 to mediate MyD88 dependent signal transduction responses. Similar to TLR4, there are reports showing TLR2 localization to endosomal compartments specifically early endosomes, lysosomes, and Rab11a$^+$ vesicles in monocytes (85). Some studies also indicate TLR2 dependent induction of type I IFN signaling from endocytic compartments (86, 87). How TLR2 is directed to such compartments is still unclear.

PRR REGULATION OF CROSS-PRESENTATION

Studies looking at the role of PRRs in cross-presentation have been largely limited to TLRs and CLRs. These receptors are well suited to regulate cross-presentation as they are present at the plasma membrane as well as along the endocytic pathway, where they encounter microbial antigen and initiate signaling to regulate adaptive immune responses such as cross-presentation. Here, we present evidence supporting regulation by these receptors.

TOLL-LIKE RECEPTORS

There are several studies that show that TLR signaling enhances cross-priming of CD8 T cells (88). The *in vivo* contribution of TLR3 to cross-priming became clear after an elegant study demonstrated that signaling via TLR3 leads to maturation of DC and therefore promotes virus-specific CD8 T cell responses (5). Another study showed that injecting mice with apoptotic vesicles derived from *M. tuberculosis* infected macrophages, activates DC via TLR2 in a MyD88 dependent manner and can cross-prime CD8 T cells, thereby protecting mice from developing tuberculosis infection (17). Moreover, by employing biodegradable microspheres for the delivery of phagocytic cargo to DC, Schlosser et al. were able to demonstrate that the presence of both TLR ligand and antigen within the same phagosome yielded efficient CTL responses as compared to when the ligand and antigen are located in separate phagosomes (89).

One caveat of the studies above is the inability to distinguish the effects of TLR signaling on cross-presentation versus cross-priming. For example, a couple of studies revealed that TLR3 and TLR9 ligands could both induce cross-presentation of OVA by DC (90, 91). This induction of cross-presentation was found to be dependent on TLR signaling as DC from *Tlr9*$^{-/-}$ and *Myd88*$^{-/-}$ mice were unable to cross-present antigen after exposure to TLR ligands. However, the authors of these studies relied exclusively on T cell activation as a measure of displayed peptide-MHC I complexes. Given that TLR signaling also controls DC maturation and expression of co-stimulatory molecules that are pivotal for T cell activation, it is difficult to rule out the confounding factor of

impaired co-stimulation and decreased DC maturation seen with TLR deficient DC. In fact, the inability of *Myd88*$^{-/-}$ DC to activate CD8 T cells after phagocytosis of virally infected cells was fully restored by treatment with a CD40 cross-linking antibody suggesting that defective cross-priming was due to impaired TLR induced co-stimulation rather than cross-presentation *per se* in this particular case (92).

The most direct way to assess cross-presentation is to use a conformation dependent antibody directly against preformed peptide-MHC I complexes on the surface of the APC. However, these antibodies are quite insensitive and work successfully only when DC were pulsed with large amounts of antigen. Nevertheless, Christian Kurts' group successfully used 25D1.16 antibody to detect SIINFEKL-MHC I complexes and thus demonstrated increased cross-presentation with TLR signaling after uptake of soluble OVA in the presence of LPS (93). This increase in cross-presentation was mediated by TLR4, MyD88 and not TRIF signaling. However, this study was focused on the cross-presentation of soluble antigen and hence whether TLR signaling enhances cross-presentation of phagocytosed particulate antigen remains to be determined.

C-TYPE LECTIN RECEPTORS

C-type lectin receptors contain at least one carbohydrate recognition domain via which they bind to sugar moieties on self or microbial derived antigens. CLRs can regulate cross-presentation, although most of them do so by regulating antigen uptake. For example, CD209 and mannose receptor have been reported to increase internalization of cargo and target antigens to early endosomes for cross-presentation (94, 95). DNGR1 was also reported to enhance cross-presentation of cellular antigens derived from necrotic dying cells (96). Interestingly, despite intact maturation phenotypes and maintenance of signals to relay co-stimulation in DNGR1 deficient DC, these DC were impaired in cross-presentation, suggesting specific regulation of cross-presentation rather than cross-priming (97). DNGR1 is also expressed at high levels in subsets of DC specialized for cross-presentation including murine CD8α$^+$ DCs and tissue-resident CD103$^+$ CD11b$^-$ DCs as well as in human counterparts BDCA3$^+$ DC (39).

CAN CROSS-PRESENTATION STILL TAKE PLACE AT STEADY STATE?

In the absence of inflammation or infection, cross-presentation of self-antigens at steady state can take place, leading to tolerance to host antigens and deletion of potentially auto-reactive CD8 T cells. Indeed, generation and activity of CTL must be tightly controlled to avoid auto-reactivity to self, given the potency of CTL in killing infected target host cells (98, 99). Here, we review existing evidence for the role of cross-presentation in both central and peripheral tolerance mechanisms.

PERIPHERAL TOLERANCE

Peripheral tolerance constitutes mechanisms of tolerance that take place after mature lymphocytes enter into the periphery. There are several studies that argue for the constitutive TLR or PRR-independent nature of cross-presentation for the induction of peripheral cross-tolerance to non-inflammatory self-antigens, leading to deletion of self-reactive CTL. Many of these models employed the expression of neo-self-antigens under the control of tissue-specific promoters like the rat insulin promoter (RIP). These models ensure that the antigens are expressed outside of the thymus, allowing researchers to specifically study peripheral tolerance.

Cross-tolerance was first demonstrated when OVA specific OT-I CD8 T cells were efficiently deleted after being adoptively transferred into a mouse expressing OVA under control of the RIP (RIP-mOVA) (100). Cross-tolerance was also shown to be important for the control of endogenous auto-reactive CD8 T cells specific for naturally expressed self-antigen (101). In this study, the authors bred the RIP-mOVA mice with mice lacking GTPase Rac1 in CD11c$^+$ cells to generate Rac1-RIP mice. Conveniently, deficiency in Rac1 GTPase selectively affected the ability of CD8α$^+$ DC to internalize antigen (33), resulting in impaired cross-presentation while leaving the classical MHC II and MHC I pathways of antigen presentation unaffected (101). Consequently, they were able to demonstrate that DC in Rac1-RIP mice failed to cross-present transgenic self-antigen and hence failed to delete transferred OT-I T cells. Moreover these mice developed symptoms of diabetes. Interestingly, mice that just had Rac1 deleted in CD11c$^+$ cells also had higher numbers of endogenous CD8 T cells, although the mice seemed healthy and did not develop autoimmunity. However, when CD25 depleted T cells from these mice were transferred into lymphopenic hosts, the hosts developed several signs of autoimmunity as a result of homeostatic T cell proliferation. Hence, the above studies clearly demonstrate the role of cross-presentation under steady state to induce peripheral tolerance.

An interesting study by Christian Kurts' group looked to see if tolerogenic DC could be converted into autoimmunogenic DC after exposure to stimulating conditions such as TLR ligands (102). TLR ligands were able to induce CTL mediated autoimmunity only in cases where antigen specific CD4 T cell help was provided concomitantly. These results demonstrated that the mere presence of TLR ligands, such as those present in commensal bacteria or those derived from the use of vaccine adjuvants, is not sufficient to break cross-tolerance mechanisms.

CENTRAL TOLERANCE

In addition to peripheral tolerance, cross-presentation was also implicated in the induction of central tolerance (103). Central tolerance is induced in the thymus where developing thymocytes that recognize peptide-MHC complexes are positively selected to express either CD4 or CD8 molecules. Subsequently, thymocytes that are able to recognize self-peptide-MHC complexes with high affinity are efficiently deleted via negative selection. Medullary thymic epithelial cells (mTECs) and DC play a critical role in mediating negative selection. mTECs exclusively express a broad range of tissue-specific antigens (TSA) (104, 105). In spite of expressing both MHC II and MHC I molecules, mTECs are poor APC. Bevan's group was the first to show that bone-marrow derived cells in the thymus were capable of cross-presenting antigen captured from mTECs (103). However, this study also showed that mTECs, by themselves, were capable of

direct presentation on MHC I molecules and were sufficient to induce CD8 T cell deletion, thus diminishing the importance of cross-presentation in central tolerance. A recent report does point to the relevance of cross-presentation by human thymic DC. Upon analyzing peptides eluted from both MHC I and MHC II molecules of human thymic DC, the authors observed that around 22% of the MHC I ligands were derived from proteins present in the vesicular/extracellular compartment the presentation of which would typically be associated with the classical MHC II pathway (30).

CONCLUSION

The studies we reviewed here certainly point to the constitutive nature of cross-presentation, however, an increasingly large body of work now provides strong evidence for the capacity to enhance cross-presentation by signals from inflammatory PRRs. Having mechanisms of regulation in place allows for the generation of robust CTL responses during an infection while maintaining induction of tolerance at steady state. Further insight into these regulatory mechanisms may potentially help in tailoring better therapeutic strategies to combat infectious agents as well as tumors, while preventing autoimmunity. Hence, elucidating the mechanistic differences in vesicular trafficking between steady state and inflammatory cross-presentation would be important for developing new rationales in the design of safe and effective vaccines for anti-viral, anti-bacterial as well as anti-tumor immunity.

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Human plasmacytoid dendritic cells: from molecules to intercellular communication network

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Plasmacytoid dendritic cells (pDCs) are a specific subset of naturally occurring dendritic cells, that secrete large amounts of Type I interferon and play an important role in the immune response against viral infection. Several studies have highlighted that they are also effective antigen presenting cells, making them an interesting target for immunotherapy against cancer. However, the modes of action of pDCs are not restricted to antigen presentation and IFN secretion alone. In this review we will highlight a selection of cell surface proteins expressed by human pDCs that may facilitate communication with other immune cells, and we will discuss the implications of these molecules for pDC-driven immune responses.

Keywords: cross talk, surface markers, T lymphocytes, viral infection, pDC migration

INTRODUCTION

Within the heterogeneous dendritic cell (DC) family, two main subsets of naturally occurring blood DCs can be discriminated based on their phenotype and functional characteristics: myeloid DCs (mDCs) and plasmacytoid dendritic cells (pDCs). The mDC subset can be further divided in $CD1c^+$ and $CD141^+$, which show a high level of similarity in protein expression yet have also specific functions in the initiation of adaptive immune responses. $CD1c^+$ mDCs have been shown to readily stimulate naïve $CD4^+$ T cells and to secrete high amounts of IL-12 in response to toll-like receptor (TLR) ligation, whereas $CD141^+$ DCs do not secrete much IL-12 but are well equipped to take up dead and necrotic cells for subsequent cross presentation of derived antigens to $CD8^+$ T cells (1–4). In contrast to mDCs, pDCs have a very different protein expression profile reflecting their important and unique function in the secretion of IFN-α and anti-viral immune response (1, 2, 5). We and others have however recently demonstrated that like mDCs, pDCs are also very well capable of presenting both soluble and particulate exogenous antigens on both major histocompatibility complex (MHC) class I and II (6). In recent years, numerous studies have been performed to characterize the expression of pathogen recognition receptors (PRRs), TLRs, Fc receptors, C-type lectin (CTL) receptors, and other surface receptors on these cells (7–13). Furthermore, these studies have emphasized both similarities and differences between DC subtypes in their cytokine release profiles, and their ability to acquire, process, and present antigens (5, 14–17). These characteristics of the different DC subtypes have recently been reviewed extensively elsewhere (2, 4, 6, 18). Here, we will focus our attention specifically on pDCs, their role in immunity and, more specifically, their (potential) direct interactions with cells of the innate and adaptive immune system via cell surface molecules. Before going into detail about these cell surface receptors and how they mediate intercellular communication, we will first give a brief summary on general pDC function and localization to provide a context in which these intercellular communications take place. Although studies on murine pDCs are numerous, and commonalities between human and murine pDCs certainly exist, major differences between pDC of both species have also been reported. Therefore, in order to prevent confusion we limited ourselves to human pDCs unless explicit stated otherwise.

pDC FUNCTION

A perturbation of the homeostatic condition that sets off the immune system can trigger either an immunogenic (immunostimulatory) or a tolerogenic (immunosuppressive) response, depending on the local circumstances and type of disease. By default, immature pDCs are tolerogenic, whereas activated (mature) pDCs can have both immunogenic and tolerogenic capacities depending on the local environment in which they are activated (19–21). pDCs are characterized as Lin^- $MHC\text{-}II^+$ $CD123\ (IL3R)^+$ $CD4^+$ $CD303(BDCA\text{-}2)^+$ $CD304(BDCA4;\ Neuropilin\text{-}1)^+$ and are mostly known for their ability to quickly produce large amounts of the Type I interferons (IFNs), IFN-α, and IFN-β, following viral infection, implicating pDCs as an important contributor during the early phase of anti-viral response (2, 22, 23).

The most important documented enveloped viruses known to stimulate Type I IFN release by pDCs are human immunodeficiency virus type 1 (HIV-1), herpes simplex virus (HSV), and influenza virus (24–27). Furthermore, parasites and bacteria containing DNA with unmethylated CpG sequences can trigger pDC activation (28–31). In addition to the anti-viral capacity,

Type I IFN release by pDCs has also been reported to be important for pDC survival, (m)DC-mediated $CD4^+$ and $CD8^+$ T cell responses, mDC differentiation, cross presentation, upregulation of co-stimulatory MHC molecules and activation of natural killer (NK), and B cells (32–34).

Because of their expression of the endosomal TLRs TLR7 and 9, pDCs, in contrast to other (immune) cells, do not need to become infected to respond to viruses or intracellular bacteria (35, 36). TLR7 recognizes guanosine or uridine-rich, single-stranded RNA from viruses or synthetic products like guanosine analogs such as R848. TLR9 senses single stranded DNA containing unmethylated CpG motifs, which are usually found in bacterial and viral genomes, and additionally senses for synthetic oligonucleotides, such as CpG-ODN (37, 38). pDCs show differential responses based on the type of virus/bacteria that is recognized, which has been suggested to be attributed to a different site of TLR activation within the endosomal system (39). For example, depending on the subtype of CpG recognized (CpG-A, CpG-B, CpG-C) the outcome of the response can be different. While CpG-A, that triggers TLR9 in early endosomes, induces IFN-α release, CpG-B, signaling from late endosomes, leads to tumor necrosis factor α (TNF-α) and IL-6 production by pDCs (40). In addition, the interplay of the various PRRs tailors the pDC response to a specific pathogenic threat. In addition to TLRs, pDCs express several CLRs, including BDCA-2, DEC-205, dectin-1 and DCIR, and Fc receptor CD32, but they lack for instance DC-SIGN (22, 41–45). Although the full repertoire of receptors is still under investigation, most of these receptors drive antigen uptake, and in concert with TLR7 and 9, coordinate pDC-mediated immune responses.

pDC LOCALIZATION

Immature pDCs circulate in the blood but have been equipped with migratory capacities as they are found within lymph nodes (LNs), tumors, and near sites of viral/bacterial infection (46, 47). At all these sites pDCs are able to promote inflammatory responses by attracting other immune cells through chemokine release, and the subsequent modulation of these cells via cytokines or direct cell–cell interactions (48–51). However, in contrast to human myeloid mDCs or murine pDCs studies, reports addressing which inflammatory chemokines and adhesion receptors specifically drive migration of human pDCs are scarce (52). Human pDCs express chemotactic receptors C-C chemokine receptor type 7 (CCR7), chemokine (C-X-C motif) receptor 3 (CXCR3), CXCR4, and ChemR23 (CMKLR1) that likely mediate migration of pDCs into lymphoid organs and/or into inflamed tissue (48, 52–55). However, due to conflicting reports the role of classical lymphoid tissue $CCR7^-$ Chemokine (C-C motif) ligand 21 (CCL21)/CCL19 pathways in resting human pDCs, is not conclusive yet (53, 56). Several studies show a high expression of CCR7 on "resting" blood DCs while others have reported a very low or a lack of expression on resting pDCs (53, 57–60). Similar to mDCs and murine pDCs, human pDCs upregulate expression of CCR7 upon TLR stimulation and migrate toward CCL21 molecules, suggesting an important role of CCR7 at least for the migration of mature pDCs to the LN (55). Furthermore IL-3 produced by T cells in the LN or by activated endothelial cells can lead to the upregulation of chemokine receptor 6 (CCR6) and CCR10 that may drive migration of activated IFN producing pDCs to inflamed skin or mucosa (61).

In contrast to mDCs, which migrate from peripheral tissue to secondary lymphoid organs via afferent lymphatic vessels, pDCs have been described to migrate to the LN mostly directly from the blood via high endothelial venules (HEVs) (62, 63). Since pDCs first need to engage and traverse the endothelial cells lining of the blood vessels, endothelial cells likely represent the first cellular contact pDCs will engage in after leaving the blood stream. pDC would require a similar migration capacity to enter into inflamed or tumor tissue, which also requires interaction with endothelial cells and extravasation. Next, within the LN, or at the site of infectious or cancerous lesions, pDCs may encounter various immune cells. In the LN, pDC have been found in close contact with T lymphocytes, Invariant Natural Killer T (iNKT) cells, B lymphocytes, and NK cells (21, 24, 42, 64–66). At sites of infection pDCs might activate or get activated by mDCs and NK cells, whereas within the tumor microenvironment pDCs are known to interact predominantly with tumor cells and regulatory T (Treg) cells (67, 68). Below we have summarized the evidence reported thus far for each of these (potential) interactions, and the circumstances under which they occur (**Figure 1**).

ENDOTHELIAL CELLS

Depending on their location (peripheral tissue or LN) and activation state, endothelial cells have been shown to express distinct cell surface molecules and to secrete a variety of chemokines and cytokines that may aid leukocyte transmigration and regulate the activation state of the migrating cells (69). Endothelial cells thus not only facilitate pDC transmigration into the site of infection, the tumor lesion, or the LN but may also have the potency to influence pDCs mediated immune responses trough pro- or anti-inflammatory cytokines as well as growth factors (69). Indeed, endothelial cells also produce IL-3 and VEGF that bind and trigger pDC marker proteins CD123 and BDCA4 respectively, and likely will promote pDC survival and migration after crossing the endothelial barrier. Documentation however of the crosstalk between human pDCs and endothelia is scarce and limited to a few recent studies that we will discuss. Intriguingly, and in contrast to murine pDCs, both resting and matured human pDCs (stimulated by influenza virus) uniquely express the receptor for chemerin, ChemR23 (48). Chemerin is present on the surface of endothelial cells in the lumen of HEVs as well as in blood vessels of inflamed tissue. The interaction between endothelial cell-bound chemerin and pDC ChemR23 seems to play a crucial role in the migration of pDC from the blood both into LNs and into inflamed tissue (**Figure 2**) (48, 70, 71). Like pDCs, T cells also migrate from the blood to the LN via HEVs and thus pDCs may exploit a similar set of molecules as used by T cells. Indeed, pDCs express adhesion molecules CD31, CD43, CD44, CD47, CD62L, CD99, and CD162 (SELPLG, CLA) that may play an important role in the tethering and rolling of pDCs on endothelial cells, but for most of these molecules, functional data for a role on human pDCs is lacking (54, 72, 73). The Lymphocyte function-associated antigen 1 (LFA-1) and very late antigen 1 (VLA-1) (CD49a/CD29) molecules might play an important role in subsequent firm adhesion and transmigration of pDCs (72). Although the expression of all these molecules was

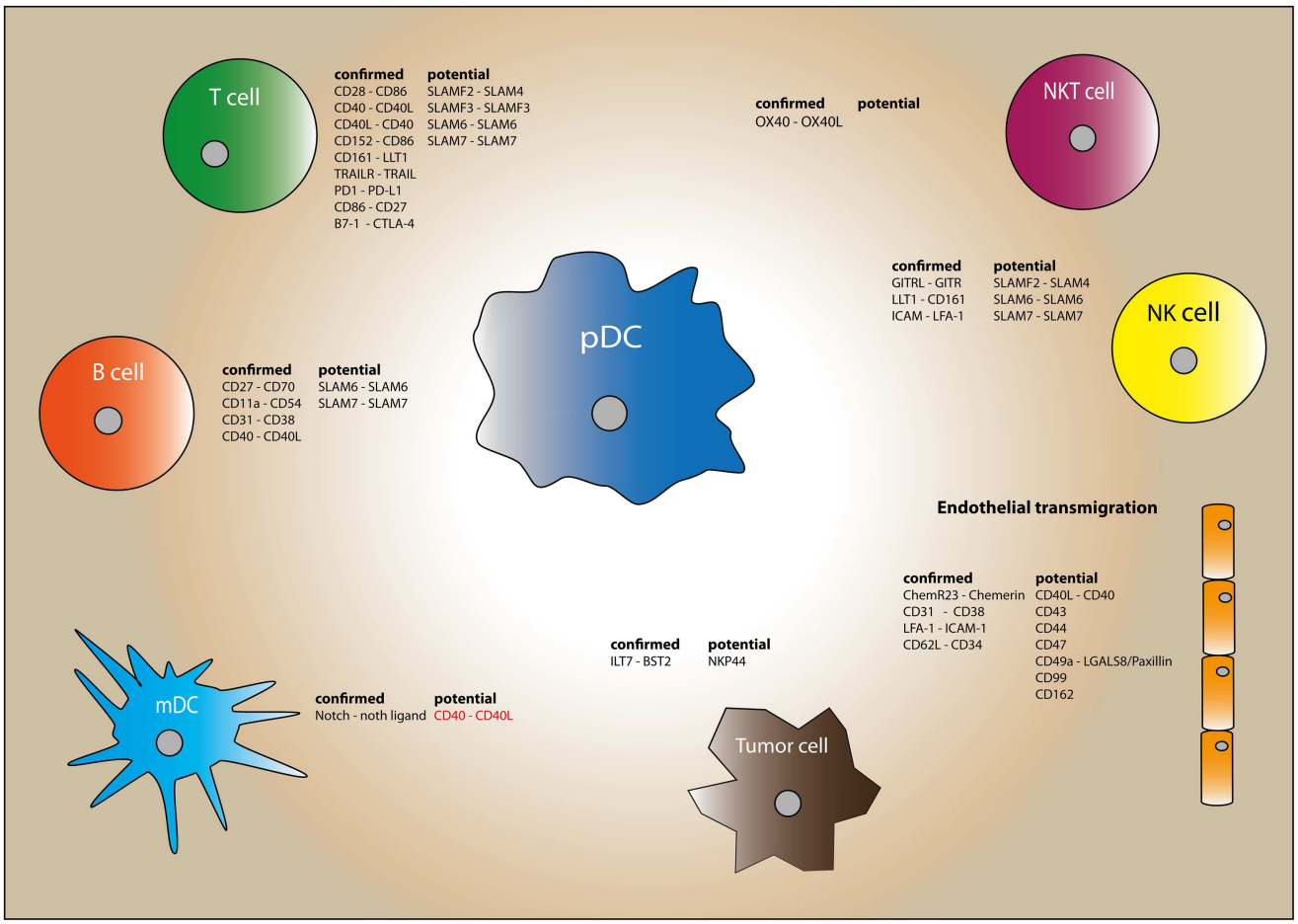
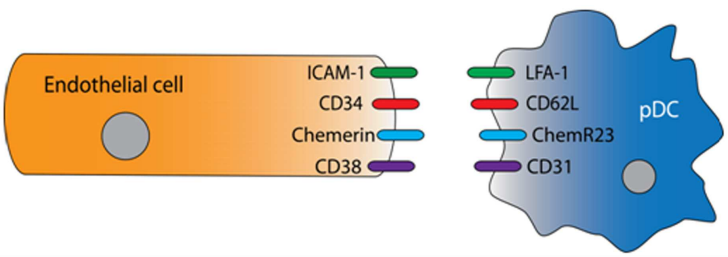


FIGURE 1 | Plasmacytoid dendritic cell have the capacity to interact with various immune cells through an array of surface molecules. The expressed surface molecules of each cell type are divided into confirmed and potential interactions. The "confirmed" molecules have been reported to have a functional effect. Molecules listed in the "potential" column are molecules that have been found on human pDC but without functional data reported in literature. Molecules playing a potential role in humans, but already confirmed with functional studies in mouse are depicted in red.



Ligand/receptor	pDC activation state	effect upon the interaction
ICAM-1 - LFA-1	resting, IL-3, HSV	Transmigration of the pDC
CD34 - CD62L	resting, downregulation upon IL-3 and HSV	Transmigration of the pDC
Chemerin - ChemR23	resting, IL-3, HSV	Transmigration of the pDC
CD38 - CD31	resting, IL-3 and HSV	Transmigration of the pDC

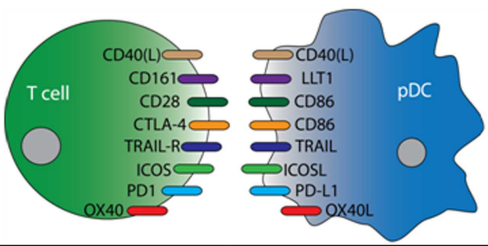
FIGURE 2 | Ligand/receptor paring of a pDC with an endothelial cell and the maturation state/activation stimuli associated with ligand or receptor expression on the pDC surface.

initially only detected by microarray, with the exception of CD44, most were confirmed by flow cytometry (74). Furthermore, flow cytometry demonstrated that expression of both CD62L (moderately) and CD99 was downregulated upon exposure to IL-3 and HSV, indicating that activated pDCs may take different migratory routes compared to their immature counterparts (74). While immature pDCs express CD62L and use HEVs to migrate into the LN, downregulation of CD62L on mature pDCs suggests that these cells enter LN without passing HEV, but rather through the lymphatic vessels. Furthermore, another study identified a cleavage of CD62L after entering the HEV suggesting this molecule may have become obsolete for pDCs following this pathway (75). In skin, in contrast, after transversing the vessel wall expression of CD62L on pDC remains high, indicating that in this case it may still have a function at a later stage (54).

In summary, although there is evidence for Intercellular Adhesion Molecule 1 (ICAM-1)/LFA, CD31/CD38, and CD34/CD62L interaction between pDCs and endothelial cells, until now, only the chemerin/ChemR23 interaction has been conclusively demonstrated to play a role during the migration process. The migratory function of the other adhesion molecules reportedly expressed by human pDC are currently only hypothetical and based on knowledge from other leukocytes or murine cells. Furthermore also the role of endothelia in the regulation of the pDC activation state awaits further study.

T CELLS

During infection, immature DCs located in the inflamed tissue get activated through pathogenic interaction and pro-inflammatory cytokines. Mature (activated) DCs subsequently translocate to the LN and induce naïve T cells to differentiate into effector T cells. Based on the repertoire of danger signals, effector T cells will have different characteristics and will evoke a different immune response. pDCs have an important role in coordinating such an immune response, since the molecules involved in the interaction between DCs and T cells determine T cell polarization (Th1, Th2, Th17). Numerous studies have established that pDCs are *bona fide* antigen presenting cells (APCs), capable of presenting exogenous antigens on both MHC class I and II molecules and thus can trigger both $CD4^+$ T helper (Th) cells and $CD8^+$ cytotoxic T cells (5, 26, 76–78). The nuances of pDCs antigen processing and presentation have recently been reviewed by Guery and Hugues (42) and Nierkens et al. (79). Here, we focus our attention on how pDC cell surface receptors may skew T cell function (**Figure 3**). Freshly isolated (immature) pDCs are known to induce $CD4^+$ T cell anergy presumably because they lack co-stimulatory molecules; conversely, activated pDC clearly induce a broad spectrum of T cell differentiation, for example, Th1, Th2, Th17, and Treg, based on the cytokines secreted and cell surface proteins expressed (21, 80–84). Like mDCs, activated pDC express high levels of MHC molecules and the co-stimulatory molecules CD80 (B7-1), CD86 (B7-2), and CD83 to present antigens and fully license and activate T cells (5,6). Several studies have demonstrated that (virally) matured pDCs, through the release of cytokines, mostly induce a Th1 phenotype (IFN-γ/Il-12 in response to CpG, virus) but Th2 (IL-4) and Th17 (IL-17) skewing has also been reported when pDC are activated with IL-3 or CD40 and TLR7 ligands, respectively (82, 85–87). Furthermore IL-21 (produced in the LN) was shown to trigger the release of Granzyme B by TLR-activated pDCs thereby dampening $CD4^+$ T cell proliferation (88). Together these studies show how pDCs may regulate immune responses. Apart from cytokines released by pDCs, several pDC surface receptors may directly affect T cell skewing and function, including the inducible T-cell co-stimulator ligand (ICOSL). pDCs express ICOSLG when activated by CpG-(A, B, and C) IL-3/CD40L or virus (Flu/HSV) (83). ICOSLG is the ligand for the



Ligand/receptor	pDC activation state	effect upon the interaction
TRAIL-R - TRAIL	HIV, influenza virus, HSVuv	TRAIL-dependent pDC-mediated killing
CD40L - CD40 (pDC)	Virus	B cell differentiation (indirect)
CD40 - CD40L (pDC)	IL-3	T cells: anti-inflammatory Th2 cytokines IL-4, IL-5, IL-10 and IL-13 pDC: no IFN-α
CD28 - CD86	CpG	T cell activation
CTLA-4 (CD152) - CD86	CpG	Th17 cell inhibition
ICOS - ICOSL	CpG-B, CpG-A HSV	IL-10 release by naive CD4+ T cells
CD161 (NKR-P1A) - LLT1	TLR activated	IFN-γ release by T cells (in combination with TCR signaling)
PD1 - PD-L1	TLR activated	IFN-γ and IL10 release by T cells
OX40 - OX40L	CpG activated, IL-3	priming of Th2 cells

FIGURE 3 | Ligand/receptor paring of a pDC with a T cell and the maturation state/activation stimuli associated with ligand or receptor expression on the pDC surface.

T-cell-specific cell surface receptor inducible costimulator (ICOS) and has been shown to trigger naive $CD4^+$ T cells to produce IL-10 during both pDC Th1 or Th2 skewing in response to CpG/virally or IL-3/CD40L-matured pDCs, respectively (83, 84). It has been suggested that ICOSL-activated pDCs generate IL-10 producing Tregs to dampen immune responses, preventing excessive inflammation (83). Furthermore TLR activated, but not resting pDCs and mDCs, express programed death receptor-ligand 1 (PD-L1), which may induce T cells anergy/suppresses T cell activation by binding to its receptor, program death ligand 1 (PD1), which is expressed by T cells (89, 90). The immunosuppressive effect of PD-L1 has been confirmed by using blocking antibodies on DCs, and additionally in follow-up studies where blocking the PD-L1/PD1 interaction lead to "enhanced tumor-specific T cell expansion and activation" (6, 91, 92). The surface receptor OX40, which is expressed on IL-3 activated pDCs, can induce a Th2 T cell response resulting in IL-4, IL-5, and IL-13 release by $CD4^+$ T cells (93, 94).

Furthermore, after stimulation either with synthetic TLR7 and 9 agonists or with the natural TLR7 agonists, like influenza virus or UV-inactivated HSV type 1(HSV_{UV}) pDCs can induce programed cell death/apoptosis, by expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (74, 95, 96). TRAIL expression on pDCs uniquely correlates with viral load, and the capacity to kill HIV-infected $CD4^+$ T cells by binding to the TRAIL receptor, a process described as "TRAIL-dependent pDC-mediated killing" (97). However, given the very limited cell numbers, it remains to be seen how important $TRAIL^+$ pDCs are in clearing a viral infection via the direct killing of infected cells (97, 98).

Another surface molecule expressed on TLR-activated pDCs that may affect T cell function is the lectin-like transcript 1 (LLT1), which in addition to activated pDCs, is expressed by most activated lymphocytes (including B cells, T cells, and NK cells) and mature monocyte-derived DCs (99). LLT1 is a ligand of CD161 (NKR-P1A), which is expressed by subsets of T cells (e.g., Th1, Th17, and a subpopulation of $CD8^+$ T cells) and NK cells. When ligated LLT1 triggers T cell proliferation and IFN-γ secretion as well as inhibition of NK cell cytotoxicity (99–102). Thus, LLT1 on pDCs may serve as a co-stimulatory molecule, and after binding to CD161 expressing T cells, could drive proliferation and IFN-γ secretion (51).

So far, we discussed how pDC receptors may affect T cell function but of course, conversely, T cells may also influence pDC function. In a multicellular immune cell signaling cascade the presentation of viral antigens by pDCs brings about IL-2 release by T cells as well as CD40L expression. T cell CD40L upon binding to CD40 on pDCs, triggers IL-6 release by pDC, which in turn enables B cell plasma blasts to become antibody-secreting plasma cells (**Figure 8**) (21, 64).

In summary, while immature pDCs predominantly induce T cell anergy, their activated counterparts may have either inhibitory or activating effects on T cells. Which of the latter in the case depends on stimuli that trigger pDC maturation and which cytokines and surface molecules are expressed as a result. Thus pDCs play pivotal role in T cell activation and fine tuning of the adaptive immune response.

iNKT CELLS

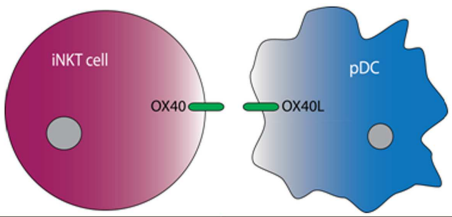
Natural Killer T (NKT) cells form a specialized T cell subset expressing a semi-invariant T cell receptor (TCR-αβ) and surface antigens traditionally associated with NK cells. The unique TCR on their cell surface enables NKT cells to recognize glycolipid antigens rather than peptides, presented in the context of the MHC class I-like molecule, CD1d (103). The most well characterized subset of NKT cells are called iNKT cells, since they express an invariant TCR-α chain, and are reactive to the potent NKT cell agonists α-galactosylceramide (α-GalCer) (103).

Studies have shown that pDCs interact with iNKT cells directly, both via cell–cell interactions and by cytokine release (104). In contrast to the mDCs, pDCs lack the expression of CD1d, which is an important molecule for crosstalk with iNKT cells (105). Nonetheless, over the past few years the ability of iNKT cell to "sense" subtle changes within their microenvironment in a CD1d-independent mechanism, uncovered that cytokines released by pDCs are essential (106, 107). Indeed, CpG activated pDCs upregulate activation markers on iNKT cells via TNF-α and IFN-α release, and selectively enhance double-negative iNKT cell survival but not that of other NKT cell populations (104). However, the interplay of iNKT cells with pDCs alone is not sufficient for iNKT expansion and does not lead to a cytokine release by iNKT cells. Rather, the CpG activated pDCs enables the iNKT cells to productively interact with CD1d expressing mDCs, thus initiating an immune response (61). Both iNKT cells and mDCs lack expression of TLR9 and are therefore unresponsive to CpG; hence, cytokines released upon ligation of TLR9 on pDCs modulate the tissue microenvironment. Not only cytokines, but also a direct interaction between pDCs and iNKT cells may be of importance; CpG-stimulated pDCs express the ligand CD252 (OX40L), which binds CD134 (OX40) present on the surface of iNKT cells, and augments IFN-γ release by iNKT cells in response to lipid antigen presentation by mDCs (**Figure 4**) (66). Further support for such a direct interaction between pDCs and iNKT cells via OX40L/OX40 comes from murine studies (108–110).

In summary, the interaction between iNKT cells and pDCs can be both via cytokines and via direct cell interaction. The role so far seems to facilitate the activation of iNKT by CD1d expressing mDCs. This may become important particularly in situation when TLR9 ligands are available. So far, only OX40L/OX40 are known to play a role in the direct pDC/iNKT cell cross talk.

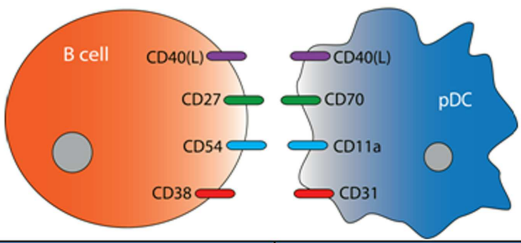
B CELLS

B cells are the only cells that produce antibodies, and therefore, have a critical role in the humoral immune response. Release of Type I IFNs by pDCs leads to an increase of TLR7 and several activation markers on B cells (111, 112). Moreover, as outlined above, pDCs, in concert with T cells, control B cell differentiation into plasma cells via the secretion of IFN-α and IL-6 (64). In addition, pDCs can affect B cells via direct cell–cell contact. Several studies have shown the importance of CD40-CD40L interactions between B cells and pDCs (**Figure 5**) (24, 64, 65). In addition, upon activation with CpG, pDCs were demonstrated to interact with B cells via CD70/CD27 molecules. This interaction results in B cell growth, differentiation, and immunoglobulin secretion (113).



Ligand/receptor	pDC activation state	effect upon the interaction
OX40 - OX40L	CpG activated, IL-3	augment IFN-γ release by iNKT cells upon additional mDC binding

FIGURE 4 | Ligand/receptor paring of a pDC with an iNKT cell and the maturation state/activation stimuli associated with ligand or receptor expression on the pDC surface.



Ligand/receptor	pDC activation state	effect upon the interaction
CD40L - CD40	HIV-1 infection	inhibition of IFN-α production
CD40 - CD40L	HIV-1 infection	inhibition of IFN-α production
CD27 - CD70	CpG	B cell growth, differentiation and immunoglobin secretion
CD38 - CD31	resting, RNA-containing IC–stimulation	IFN-α production by pDCs
CD54 - CD11a	resting, IL-3 and HSV	IFN-α production by pDCs

FIGURE 5 | Ligand/receptor paring of a pDC with a B cell and the maturation state/activation stimuli associated with ligand or receptor expression on the pDC surface.

Furthermore, recent *in vitro* studies have shown that activated B cells are able to stimulate matured pDC to produce IFN-α by direct cell–cell contact (114). Blocking the surface molecules OX40L, CD27, CD40, or CD40L with monoclonal antibodies did not influence the effect of B cells on pDC-derived IFN-α production. However, the IFN-α production by pDCs was significantly reduced when blocking LFA-1 or PECAM-1 (CD31) by 50% and 80%, respectively, indicating that these molecules are at least partially responsible for B cell mediated pDCs activation (114).

Taken together, pDCs and B cells are able to induce reciprocal cytokine release and activation by both soluble mediators and direct cell–cell interaction, and so far have been found to be predominantly stimulatory in nature (64, 113, 114).

mDCs

Synergism of mDCs and pDCs are not restricted to the activation of NKT cells. pDCs and mDCs have been demonstrated to be in close contact *in vivo* at steady state as well as under inflammatory conditions, and it has been suggested that they act synergistically to induce more potent immune responses (115–117). Upon stimulation both mDCs and pDCs function as APCs and follow a similar maturation program, and express the co-stimulatory markers CD40, CD80, CD83, and CD86 to interact with T cells (17, 118). However, there are complementary differences especially in the expression of PRRs (e.g., TLRs, CLRs) and thus in their response to pathogenic triggers. Whereas mDC subtypes express TLR1, 2, 3, 4, 5, 6, 8, and 10, but no TLR7 and 9, the expression of these TLRs on pDCs is the exactly opposite except from TLR2 and 10, which are shared (7, 8, 35, 119–122). pDCs respond to TLR7 and 9 ligands with large amounts of IFN-α and TNF-α (123). In contrast, mDCs release very different cytokines, primarily IL-1β, IL-8, IL-6, IL-10, IL-12p70, TNF-α to variable extents, upon triggering of their TLRs (7, 118, 122). Upon viral infection pDCs are known to respond quicker and with larger amounts of cytokines than mDCs (124). Thus pDCs and mDCs have non-overlapping sensitivities to invading pathogens, and accumulating reports suggest that pDC and CD1c-mDC may cross-activate each other for a more effective immune response. Crosstalk may occur in a paracrine fashion through cytokines like Type I IFNs and TNF-α but also via direct cell contact (118, 125). In a paracrine fashion TNF-α expressed by pDC can cross-activate co-cultured CD1c-mDCs (126). However, there is clear evidence to suggest that CD1c-mDCs and pDCs in other cases require close contact for some parts of this crosstalk, until now it is unclear what molecules are involved (118).

Recent studies by Piccioli et al. implies that several members of the TNF family, CD40L/CD40, OX40L, HEVML, RANKL, CD27, CD30L, glucocorticoid-induced tumor necrosis factor receptor-ligand (GITRL), and 4-1BB are redundant in the CD1c-mDC/pDC cross talk (9). Experimental evidence for the absence of a role for any of these interactions however was so far not reported but only came from unpublished blocking experiments mentioned in these studies, making it extremely hard to deduce whether these interactions can and should be excluded completely (9, 118). Interestingly, murine models do suggest that the TNF member CD40/CD40L may have a crucial role in the CD1c-mDC/pDC cross talk, yet this result needs to be recapitulated in human CD1c-mDC/pDC assays (9).

So far only for the NOTCH receptor-ligand interaction evidence is provided for a role in the communication between pDCs and CD1c-mDCs but again experimental evidence is scant (**Figure 6**) (117). With co-culture experiments they demonstrated that LPS-activated CD1c-mDCs caused an upregulation of maturation marker (CD25, CD86) on the pDC surface and increased IL-6 and CCL19 release in the supernatant. To confirm the involvement of NOTCH pathway, experiments with γ-secretase/NOTCH inhibitor DAPT and soluble NOTCH ligands were preformed and showed a reduced effect on Notch target genes. Activation of the NOTCH pathway upon CD1c-mDC/pDC interaction suggests that this intercellular contact promotes an immune stimulatory response, however, further experiments are needed to unravel the exact mechanism and other molecules potentially involved in this CD1c-mDC/pDC cross talk (117).

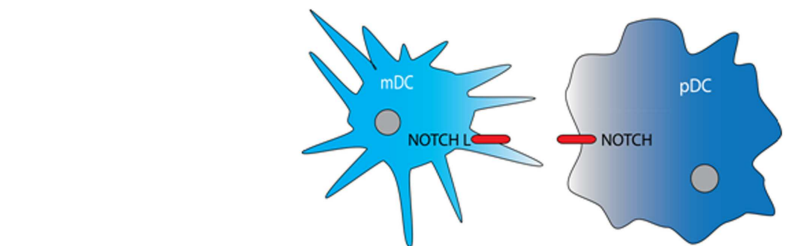
Another possible candidate for the interaction between pDC and CD1c-mDC is ICAM-1, expressed on both, pDCs and mDCs, and known as an widespread adhesion molecule with co-stimulatory activity on other immune cells (127). ICAM-1 was found to be strongly upregulated on pDC upon stimulation with TLR9 ligand CpG, while its matching receptor LFA-1 (CD11a/CD18) is constitutively expressed on CD1c-mDCs (9).

Taken together, there is clear evidence that direct cellular interactions are indeed important for CD1c-mDC/pDC cross talk in humans, similar to what was observed in murine studies. However, besides NOTCH receptor-ligand interactions, any experimental evidence that argues in favor of or against the involvement of other specific receptor-ligand interactions is so far lacking (9, 118).

NK CELLS

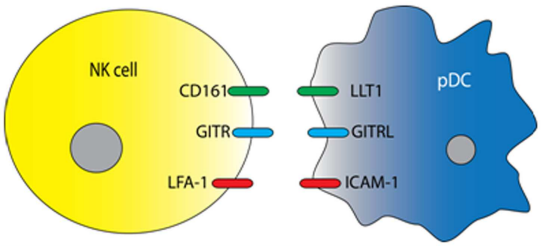
Natural killer cells belong to the innate immune system and are able to respond rapidly to virally infected cells and to tumor formation. This is due to their unique ability to recognize stressed cells or the absence of MHC on the surface of infected or malignantly transformed cells, and their subsequent ability to lyse these cells. The bi-directional pDC-NK cell interaction is known to play an important role in host defense, and again is mediated both by cytokines and via direct cell contact (128, 129). Type I IFNs secreted by pDCs have long been known to enhance the cytolytic potential of NK cells, and NK cells co-cultured with pDCs are more activated, and have increased cytolytic activity (49, 50, 130–133). pDCs and NK cells have been found in close proximity in the T cells areas of human tonsils (50). In addition, during infection or in case of a malignancy, pDCs and NK cells may migrate simultaneously to the site of the lesion, for example during Herpes simplex infection (25). These reports demonstrate the ample opportunities for these cells to engage in direct interactions, which is further supported by the findings that, when co-cultured, pDCs and NKs cells readily interact (134). Upon stimulation by a virus or CpG, pDCs express GITRL that can bind GITR expressed by NK cells (**Figure 7**). Via the (GITRL)-GITR interaction mature pDCs enhance NK cell mediated killing as well as IFN-γ production. To affect NK cells, however, pDCs expressing GITRL do require the simultaneous presence of IFN-α (50). Furthermore, while the upregulation of CD69 on the surface of NK cells depends on the release of IFN-α and TNF-α by mature pDCs, upregulation of HLA-DR on the surface of a subpopulation of NK cells depends on direct pDC-NK cells contact (49). HLA-DR expressed on NK cells is thought to play an important role in handling bacterial infections such as *Mycobacterium bovis* (BCG) (135). Although the interaction responsible for HLA-DR upregulation remains to be elucidated, it is known that the maturation state of the pDC is not important for the induction of HLA-DR expression on NK cells, indicating the HLA-DR inducing factor is not affected by pDC maturation (49, 130, 132, 136).

The bi-directional crosstalk between pDCs and NK cells also affects pDC function; IL-2, immune complex or IL-12/IL18-stimulated NK cells induce pDCs to release IFN-α which was shown to depend largely on LFA-1-mediated interactions between NK cells and pDCs, and to a lesser extent on NK cell secreted



Ligand/receptor	pDC activation state	effect upon the interaction
NOTCH ligand - NOTCH	IL-3	possible T cell recruitment and activation

FIGURE 6 | Ligand/receptor paring of a pDC with a mDC and the maturation state/activation stimuli associated with ligand or receptor expression on the pDC surface.



Ligand/receptor	pDC activation state	effect upon the interaction
CD161 (NKR-P1A) - LLT1	TLR activated	LLT1 on target cells inhibits NK cell cytotoxity
GITR - GITRL	CpG	NK cell mediated killing and IFN-γ release
LFA-1 - ICAM-1	RNA contain. imm. complexes	IFN-α production by pDCs

FIGURE 7 | Ligand/receptor paring of a pDC with an NK cell and the maturation state/activation stimuli associated with ligand or receptor expression on the pDC surface.

MIP1α (132, 134). LFA-1 and FcγRIIIA on the pDC also increase cytokine release by NK cells (137). Furthermore, IL-2 stimulated NK cells induced pDCs to express the maturation marker CD83, but not CD80 and CD86, in a contact dependent manner, which also indicates the existence of different stimulatory pathways that can induce expression of different maturation markers on pDCs (132).

Contact with NK cells potentially puts pDCs in danger of becoming lysed. However, immature pDCs are protected from NK cell mediated lysis, and this is at least partly due to the high expression of HLA class I, and the absence of Nectin-2, the ligand for NK cell activating receptors DNAM-1 (132, 138). Culture of pDCs with IL-3 however causes the upregulation of Nectin-2 on pDCs, and makes them more susceptible to DNAM-1 and NKp30-mediated killing (138). Activation of pDCs by TLR7 and 9 may help to prevent NK cells lysis as they express the LLT1 (LLT1 or CLEC2D; above), which is a ligand of NK cell surface protein P1A (NKR-P1A; CD161). P1A is expressed by both NK and NKT cells and when ligated inhibits NK cell cytolytic function and IFN-γ release (51, 99, 101, 139). Taken together, pDCs in various modes of action, seem to be differentially susceptible to NK cell-mediated lysis through the absence of activating NK cell receptor-ligands, as well as the regulated expression of ligands for NK cell inhibitory receptors. Also, high MHC I expression is protective.

In summary, non-lethal pDC/NK cell interactions seem to play an important role in enhancing the early immune response to a viral or bacterial infection as pDCs activate the NK cell by producing IFN-α and via GITRL. This feed-forward system likely promotes NK cells to rapidly lyse infected cells (132). NK cell activity in turn induces a further increase of IFN-α by pDCs and promotes their maturation, which may in turn increase the recruitment and survival of mDCs (**Figure 8**).

TUMOR CELLS

Several early studies have reported decreased numbers of pDCs and mDCs in the blood of patients suffering from various types of cancers (140–143). However, a recent study with melanoma patients detected no significant difference between the levels of immature pDCs in healthy donors and patients (144). Compared to healthy volunteers, pDCs derived from melanoma patients did, however, show a higher expression of CCR6, and increased ability to migrate toward Chemokine (C-C motif) ligand 20 (CCL20), a ligand for CCR6 (144). CCL20 is expressed by keratinocytes in the skin and by melanoma cells, suggesting that the CCL20/CCR6 interaction is involved in the pDC migration process from the blood to the tumor (145–147). Indeed, high pDC infiltration have been observed in many types of cancer including melanoma, head and neck cancer, ovarian and prostate cancer, and these infiltrates mostly negatively correlate with patient survival. On the other hand, an increase of pDCs in tumor-draining LN may be beneficial [reviewed in (143, 148)]. pDCs infiltrated in tumor microenvironment are mainly immature, and therefore seem to be predominantly immunosuppressive/tolerogenic (148). In recent years, evidence has accumulated that tumors may block anti-tumor response by maintaining pDCs in an inactive/tolerogenic state. Mechanisms responsible for keeping the pDC in this state include the secretion of prostaglandin 2 (PGE_2) and TGF-β, which, in a synergistic manner inhibit pDC-derived IFN-α and TNF-α production in response to TLR7 and 9 ligands, as well as inhibiting CCR7 expression, thereby impairing the migration of pDCs to the tumor-draining LN to prime T cells with tumor antigens (148–150). In addition, there is evidence that PGE_2-stimulated pDCs indirectly support tumor cell proliferation, migration, and invasion, as well as tumor angiogenesis, via the release of IL-6 and IL-8 (151–158). Furthermore, tumor-resident pDCs may also influence tumor growth indirectly through the induction of Tregs. In epithelial ovarian cancer the majority of $Foxp3^+$ Treg cells accumulating in the tumor microenvironment expressed the ICOS, and tumor pDCs expressing the ICOSL were shown to be essential for the expansion and suppressive function of these regulatory $Foxp3^+$ Tregs (67, 68).

On their surface, unstimulated pDCs (uniquely with respect to all other leukocytes) express the immunoglobulin-like transcript 7 (ILT7) protein, which is activated by binding to bone marrow stromal cell antigen 2 (BST2, CD317; reviewed in (159)). BST2 is expressed on human cancer cells, monocytes, and vascular endothelium in response to IFN-α (**Figure 9**) (160–162). Similar to BDCA-2, ILT7 forms a complex with FcεRIγ, which, when ligated activates an immunoreceptor tyrosine-based activation motif (ITAM)-mediated signaling pathway that dampens

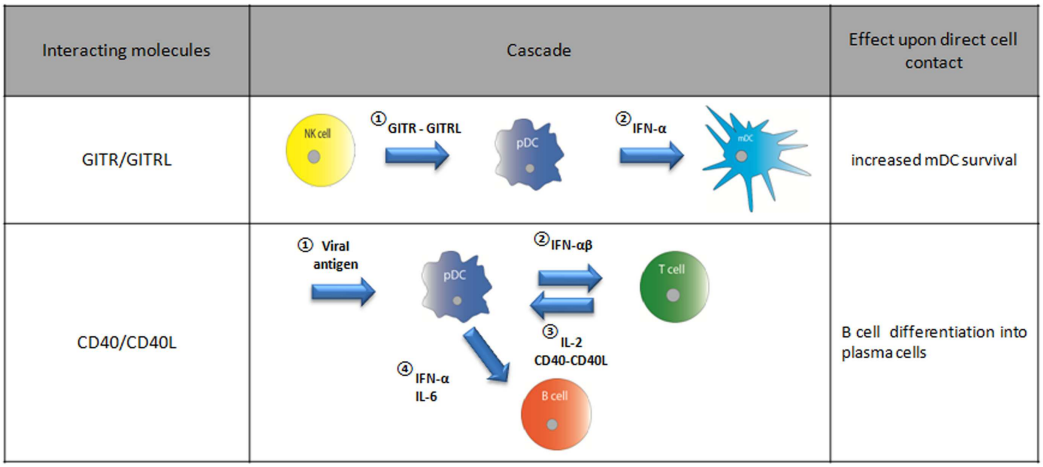
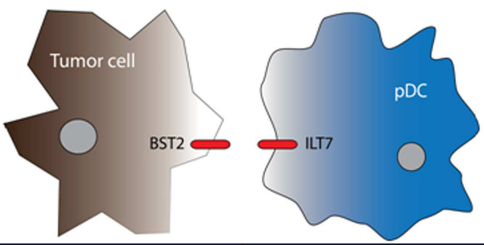


FIGURE 8 | Direct cell interaction dependent on GITR/GITRL or CD40/CD40L binding and their effect on a certain cell type.



Ligand/receptor	pDC activation state	effect upon the interaction
BST2 - ILT7	resting downregulation upon IL-3, HSV and CpG	immune tolerance

FIGURE 9 | Ligand/receptor paring of a pDC with a tumor cell and the maturation state/activation stimuli associated with ligand or receptor expression on the pDC surface.

TLR-7 and 9-induced IFN-α and TNF-α production (163, 164). ILT7 is downregulated upon stimulation of the pDCs by CpG, HSV, or IL-3, suggesting that pDC maturation prior to entering the tumor site may partly protect it from this suppressive mechanism (159, 164, 165). In addition to ILT7, immature pDCs also express the ITIM motif containing receptors ILT2 and ILT3 that bind to MHC class I molecules, and an unknown ligand, respectively. Both receptors are associated with immune tolerance, probably through the suppression of T cell responses, and in agreement with this notion, these receptors are downregulated upon pDC activation (166–168). Whether these molecules may also have an active role in the pDC-tumor cell interaction, or rather regulate pDC-T cell activation, remains to be investigated. Likewise, there may be a yet unappreciated role for pDC expression of NKp44, which has been demonstrated to down modulate pDC IFN-α responses upon ligation, and may be utilized by the tumor to dampen pDC-mediated immune responses (169). Indeed, an inhibitory NKp44 ligand complex containing HLA I and PCNA was recently reported to be expressed by several tumor cells (159, 165, 170).

So far, most studies point out an immune suppressive role for pDCs favoring tumor progression, however several other studies demonstrate that the situation may be very different if pDCs are properly activated. In this case pDCs may trigger anti-tumor T cell-mediated immune responses (above) or even actively kill tumor cells (171, 172). As previously discussed, in relation to the T cell/pDC interaction, activated pDCs express TRAIL, which induces an apoptotic process by binding to the TRAIL receptors. Tumor cells are known to be sensitive to TRAIL, and via this interaction could directly induce tumor cell apoptosis (171). Avoiding this apoptotic pathway by downregulation of TRAIL has been reported for several cancers by numerous studies (173).

Taken together, the suppressive tumor microenvironment decreases the immune stimulatory functions of pDCs resulting in tumor progression. Preventing these processes, while at the same time activating pDCs, forms a promising target for anti-tumor therapies. Moreover, in a recent clinical feasibility study in our department, we demonstrated that the administration of

autologous *ex vivo*-matured tumor antigen loaded pDCs proved to be successful and induced objective clinical responses in several patients (60).

LESS EXPLORED IN pDC CELLULAR INTERACTIONS: THE SLAM FAMILY

Above we have provided an overview of the interactions pDCs likely engage in during their lifecycle, as based on experimental evidence and *in vivo* proximity. In addition we discussed the molecules likely to participate in these interactions. There is, however, a poorly understood family of proteins which are highly expressed pDCs, and which deserve more attention. This is the SLAM family of receptors, for which a role in a diverse range of cellular interactions is highly suspected, yet currently unexplored. Five family members are expressed on the pDC surface (largely) independent of its activation state: SLAMF2 (CD48; BLAST1), SLAMF5 (CD84), SLAMF3 (CD229; Ly9), SLAM7 (CD319, CRACC), and NTBA (CD352) (74, 102). Except for SLAMF2 (below), these proteins have in common that they engage mostly in homotypic interactions; such interactions may occur between homotypic cells, but also between different cell types, opening up the possibility that these molecules mediated direct cell interactions of pDCs with each other, or with others cells also expressing these receptors (174). Homotypic SLAM family interactions have the ability to regulate cell activation and proliferation as well as cytolytic activity (174). SLAMF5 is highly expressed by many immune cells, and has been shown to play a role in T cell-B cell adhesion, and for optimal germinal center formation (175, 176). Furthermore SLAMF5 was detected on leukemic pDCs, and can work as an inhibitor for FcεRI-mediated signaling in mast cells (177, 178). SLAMF3 is also expressed on T cells. Here it reduces IFN-γ production and ERK activation upon stimulation, and thus via this molecule pDCs might trigger a similar response (175, 179). SLAM7, in contrast, is widely expressed on activated B lymphocytes, NK cells, and $CD8^+$ T lymphocytes (74, 180–182) and has been shown to promote B cell proliferation, activate NK cell cytotoxicity (but not NK cell proliferation) (180, 182, 183). Finally, NTBA in addition to pDCs, is present on NK, T, and B cells where it may affect cytotoxicity as well as the IFN-γ and TNF-α release (174, 184, 185). Interactions between NTBA on pDCs and NK cells may therefore have the potential to positively regulate both NK cells and pDCs.

In contrast to the other family members, SLAMF2 which is also present on the surface of pDCs, engages in a heterotypic interaction with family member 2B4 (CD244), and could thus play a role in the interaction of pDCs with 2B4-expressing NK or T cells (74, 175, 186). SLAMF2 via 2B4 can activate NK cells (186).

Overall, although experiments are largely lacking, the presence of such a high number of SLAM family members on pDCs, their homotypic interactions, as well as the known effects of their triggering on other immune cells makes us speculate that pDCs may very well exploit these receptors to communicate with other immune cells.

CONCLUSION AND OUTLOOK

In this review we summarized the existing data which supports the idea that during their life cycle, human pDCs interact with numerous immune cells. Additionally, we have attempted to provide a contemporary overview of the molecules that drive these interactions, and the consequences of their expression on pDCs. It is clear that pDCs play a pivotal role in ensuring a rapid immune response, especially upon viral infection, by strong IFN-α release, but also via direct cell–cell interaction. Depending on the pDC activation state, cytokines released by pDCs and direct pDCs surface receptors may inhibit or activate other immune cells. This large influential capacity of pDCs suggest that they are master regulators of both innate and adaptive immune responses. Besides secretion of the highly potent yet broadly acting IFN-α, pDCs have a highly versatile repertoire of cell surface molecules to further fine tune immune responses. These characteristics make them an interesting and potential highly valuable therapeutic target to be exploited or targeted in cancer therapy, infectious or autoimmune disease. Importantly, controlling cytokine secretion and the surface expression of specific receptors is essential to steer the immune response into the desired direction. In cancer therapy, lifting the suppressive actions of tumor-resident pDCs may greatly enhance existing/endogenous anti-tumor immune response (187). In addition, anti-cancer immune responses may be initiated or boosted by vaccination with autologous tumor antigen loaded pDCs (60). A preliminary clinical trial using pDCs in melanoma vaccination therapy, carried out by our department, has demonstrated the use of thick born virus vaccine (FSME)-matured pDCS in cancer vaccination is safe and feasible, and despite low patients numbers, showed an improved survival of pDCs vaccinated patients (60). In particular, the extremely low doses ranging from 0.5 to 3 million pDC per patient demonstrate the potency of these cells. The exact reason for the success of pDCs however is not yet completely understood, but in addition to IFN-α production and the induction of tumor-specific T cells, we envision there may also be a significant role for the effective combination of surface receptors expressed by the pDCs and their resulting interaction with other immune cells.

In conclusion, research over the past few years has greatly increased our knowledge of the repertoire of pDC-expressed surface receptors and cellular interaction partners, and has emphasized that there is more to the pDCs than IFN-α alone. Importantly, however, further studies are required to identify the role of these molecules and interactions in pDC function and immune responses in general.

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Combinatorial cytokine code generates anti-viral state in dendritic cells

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The physiological function of the immune system and the response to therapeutic immunomodulators may be sensitive to combinatorial cytokine micro-environments that shape the responses of specific immune cells. Previous work shows that paracrine cytokines released by virus-infected human dendritic cells (DC) can dictate the maturation state of naïve DCs. To understand the effects of paracrine signaling, we systematically studied the effects of combinations cytokines in this complex mixture in generating an anti-viral state. After naïve DCs were exposed to either IFNβ or to paracrine signaling released by DCs infected by Newcastle disease virus (NDV), microarray analysis revealed a large number of genes that were differently regulated by the DC-secreted paracrine signaling. In order to identify the cytokine mechanisms involved, we identified 20 cytokines secreted by NDV infected DCs for which the corresponding receptor gene is expressed in naïve DCs. By exposing cells to all combinations of 19 cytokines (leave-one-out studies), we identified five cytokines (IFNβ, TNFα, IL-1β, TNFSF15, and IL28) as candidates for regulating DC maturation markers. Subsequent experiments identified IFNβ, TNFα, and IL1β as the major contributors to this anti-viral state. This finding was supported by infection studies *in vitro*, by T-cell activation studies and by *in vivo* infection studies in mouse. Combination of cytokines can cause response states in DCs that differ from those achieved by the individual cytokines alone. These results suggest that the cytokine microenvironment may act via a combinatorial code to direct the response state of specific immune cells. Further elucidation of this code may provide insight into responses to infection and neoplasia as well as guide the development of combinatorial cytokine immunomodulation for infectious, autoimmune, and immunosurveillance-related diseases.

Keywords: TNFa, IL1b, IFNb, anti-viral signaling, DC maturation, combinatorial effect

INTRODUCTION

The limitations of single cytokine therapy have motivated interest in evaluating the effects of combinatorial treatment. Individual therapeutic cytokines often fail to achieve full or sustained clinical benefits for many patients. For example, IFNα, which is the current therapy for chronic hepatitis C infection, fails to clear HCV titers in half of treated patients (1). The cytokine interferon beta (IFNβ) has limited activity against multiple sclerosis in a large segment of patients (2). Cytokine combination therapy, where two or more cytokine-based medications are simultaneous administered to treat a single disease, has shown promise in multiple medical conditions, such as cancer (3), myocardial infarction (4), and osteoporosis (5). Recent studies have also begun to reveal how combined extracellular stimuli can synergistically direct the responses of immune cells. Retinoic acid combined with IL-15 causes dendritic cells (DCs) to skew the T-cell polarization toward TH17 cells (6). SCF and IL-2 have a synergistic effect on the proliferation NK cells (7). TNFα and IFNγ act together on smooth airway cells to enhance CXCL-10 expression (8). IL17 together with TNFα or IL1β induces MCP-1 and MIP-2 in murine mesangial cells (9). Despite its potential, studying combinations of cytokines is experimentally difficult and relatively little systematic exploration in this important area has been reported.

We previously reported that paracrine signaling mediated by the complex mixture of cytokines secreted by virus-infected DCs in culture causes naïve uninfected DCs to develop an anti-viral state characterized by upregulation of DC maturation markers, increased phagocytic activity, and greater resistance to viral infection (10). Since the discovery of type I interferon, paracrine cytokine signaling has been recognized as a crucial component in orchestrating the immune responses to virus infection. However, IFNβ pretreatment alone is not sufficient to induce this paracrine induction of anti-viral activated DCs (10). In the present study, we investigate the combinatorial cytokine code underlying this effect, by studying combinations of the single components of the secretome of virus-infected DCs. Understanding how this combinatorial cytokine code modulates immune responses may guide the development of better combination therapy approaches and help elucidate how the microenvironment directs appropriate responses in specific cell types during infection.

MATERIALS AND METHODS

DIFFERENTIATION OF DCs

All human research protocols for this work have been reviewed and approved by the IRB of the Mount Sinai School of Medicine. Monocyte-derived DCs were obtained from healthy human blood donors following a standard protocol described elsewhere (11). Briefly, human peripheral blood mononuclear cells were isolated from buffy coats by Ficoll density gradient centrifugation (Histopaque, Sigma Aldrich) at 1450 rpm and $CD14^+$ monocytes were immunomagnetically purified by using a MACS CD14 isolation kit (Miltenyi Biotech). Monocytes were then differentiated into naïve DCs by 5–6 days incubation at 37°C and 5% CO_2 in DC growth media, which contains RPMI Medium 1640 (Invitrogen/Gibco) supplemented with 10% fetal calf serum (Hyclone), 2 mM of L-glutamine, 100 U/mL penicillin and 100 g/mL streptomycin (Pen/Strep) (Invitrogen), 500 U/mL hGM-CSF (Preprotech), and 1000 U/mL hIL-4 (Preprotech). All experiments were replicated using cells obtained from different donors. Overall, we used DCs from 21 different donors for this study.

VIRUS PREPARATION AND VIRAL INFECTION

The Newcastle disease virus (NDV) (rNDV/B1) was generated in Prof. Peter Palese's laboratory (12). NDV-RFP, Influenza A/California/04/09 (H1N1), and A/Puerto Rico/8/1934 (H1N1) were obtained from Prof. Adolfo Garcia-Sastre's laboratory (13). For infection, virus stocks were diluted in serum free medium and added directly onto pelleted DCs at a multiplicity of infection of 1.

GENERATION OF AVDCs

Anti-viral activated dendritic cells (AVDCs) were generated by employing a trans-well system. The trans-well system consists of an upper and a lower chamber separated by a 0.4 μm PET membrane (Millipore) that allows diffusion of cytokines and chemokines through the membrane but avoids the interaction of the cells in both chambers. To generate the AVDCs, naïve DCs were infected as described above. After the 40 min incubation, the cells were washed with PBS, and cultured in the trans-well system. Infected and non-infected DCs were allocated in the upper and lower chamber, respectively. Two independent wells were set-up with infected or naïve non-infected DCs as positive and negative controls. The cultures were incubated at 37°C in 5% CO_2 for 18 h. All cells were then washed in PBS and harvested for flow cytometry analysis and RNA isolation. The supernatant was kept at −80°C for ELISA analysis of cytokines/chemokines.

MICROARRAY ANALYSIS

Samples from AVDCs, DCs infected with NDV, and DCs treated with IFNβ for 8 h were used for microarray analysis. Naive DCs served as negative control. Three samples were taken per treatment. RNA was extracted with the RNeasy plus kit (Qiagen) following the manufacturer's protocol. Gene expression was assayed using broad human genome specific HG-U133_Plus_2 GeneChip expression probe arrays (Affymetrix). Raw data was processed with the Partek Pro software using the RMA background correction, with an adjustment of GC content as pre-background adjustment. Data was normalized to its quantile, data was log transformed to a base of two, and probe sets were summarized to its mean. Principal component analysis (PCA) of samples plotted in genespace was performed for all probe sets. Robustness of the PCA was tested by randomization (Figure S1 in Supplementary Material). One-way ANOVA was calculated by using Method of Moments (14). Fisher's least significant difference with FDR as multiple testing correction was used to calculate the following contrasts AVDC vs. IFNβ, AVDC vs. CTRL, IFNβ vs. CTRL, NDV vs. CTRL. List were generated by a fold change and *p*-value (FDR adjusted) criteria. Bioinformatic analysis was performed using Ingenuity software. The data used are deposited in NCBI's gene expression omnibus (15) and are accessible through GEO series accession number GSE52081 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52081).

ELISA

In order to minimize the supernatant volume to assay, a Beadlyte Human Multiplex ELISA analysis (Millipore) was used following manufacturer instructions. Briefly, 100 μl from each compartment/well was incubated in a 96-well filter PVDF 1.2 μm plate specially designed to retain cytokines/chemokines, with a mixture of anti-cytokine IgG conjugated beads for the different cytokines/chemokines assayed. After 2 h incubation, the plate was filtered and washed three times with Assay solution (PBS pH 7.4 containing 1% BSA, 0.05% Tween-20, and 0.05% sodium azide). The washes were followed by 1.5 h incubation with biotin-conjugated anti-cytokine IgG. After Assay solution washing, Streptavidin–Phycoerythrin, was added followed by addition after 30 min Stop solution [0.2% (v/v) formaldehyde in PBS pH 7.4]. The plate was then filtered and each well resuspended in 125 μl of Assay buffer, and read in a Luminex 100 machine. Single cytokine ELISA (IFNβ) was also performed according to manufacturers protocol (PBL).

CYTOKINE TREATMENTS

Dendritic cells were exposed to 1.3 μg/mL TNFα (Symansis), 9 μg/mL CCL3 (Symansis), 3.8 μg/mL IL8 (Symansis), 20 μg/mL CXCL10 (Peprotech), 0.5 μg/mL CCL5 (Peprotech), 9 μg/mL IL6 (Peprotech), 2.8 μg/mL IFNα2 (PBL InterferonSource), 0.03 μg/mL CXCL12 (Peprotech), 2 μg/mL IFNALPHA16 (PBL InterferonSource), 0.03 μg/mL IL12a (Symansis), 4.4 μg/mL IL18 (R&D SYSTEMS), 0.2 μg/mL IL1a (R&D SYSTEMS), 1 μg/mL IL1RA (R&D SYSTEMS), 4 μg/mL IL28a (AbD Serotec), 4 μg/mL IL29 (R&D SYSTEMS), 0.1 μg/mL TNFSF15 (AbD Serotec), 0.1 μg/mL TNFSF4 (R&D Systems), 0.1 μg/mL TNFSF10 (R&D Systems), 0.2 μg/mL IL1β (eBiosciences), and 8.89 μg/mL IFNβ (PBL InterferonSource) in various combinations for 8 h. For the first screening with 20 cytokines, we used cells from three different donors. To adjust for overall differences between individuals, we normalized data of each individual to the median of all treatments. All other cytokine screening experiments were carried out with replicated from the same donor and then repeated with at least two additional donors.

FLOW CYTOMETRY ANALYSIS

Cells were washed with FACS staining buffer (Beckman Coulter) and stained with monoclonal antibodies for HLA-DR and CD86 (BD Biosciences). NDV-GFP cells were analyzed without

any additional staining. Cells were assayed on an LSRII flow cytometer (BD Biosciences) and analyzed with Cytobank software (16). Raw data as well as analyses can be downloaded at: https://www.cytobank.org/cytobank/experiments?project=565

IMAGING FLOW CYTOMETRY ANALYSIS OF BEAD UPTAKE AND APOPTOSIS

For analysis for apotosis and infectivity cells were fixed after treatment with 1% paraformaldehyde (Electron Microscopy Science), permeabilized with Methanol (Sigma), and washed in PBS and stained with influenza NP specific antibodies (Abcam) and Hoechst 33342 (Invitrogen) as nuclear dye. Single cell images were acquired using the IS 100 Imaging flow cytometer (Amnis). Apoptotic cells were identified by fragmentation of nucleus (intensity of nuclear image at a 30% threshold) and shape of the bright-field image (contrast) using IDEAS software (Amnis). To detect phagocytosis, 1 μm 488 nm fluorescence labeled latex microspheres (Polysciences Corp.) at a concentration of 50 beads per cell were co-cultured for 2 h at 37°C with cytokine pretreated cells. Single cell images were acquired using extended depth field imaging distortion in order to identify beads in different focal planes within a cell. The numbers of beads incorporated by cells were quantified in the images captured using image analysis software (IDEAS Software, Amnis Corp).

REAL-TIME PCR

mRNA expression levels were quantified by real-time reverse transcriptase polymerase chain reaction (PCR). RNA was isolated from cells using Qiagen Micro RNeasy kit following the manufactures protocol (QIAGEN). cDNA was synthesized from total RNA with AffinityScript™ Multi-Temp RT (Stratagene) with oligo dT_{18} as primer. For real-time PCR PlatinumTaq DNA polymerase (Invitrogen) and a SYBR green (Molecular Probes) containing buffer were used. The real-time PCRs were performed using a thermocycler (ABI7900HT; Applied Biosystems) as previously described (21). The RNA levels for the house keeping genes ribosomal protein S11, tubulin, and β-actin were also assayed in all samples to be used as an internal controls. mRNA measurements were normalized using a robust global normalization algorithm. All control crossing threshold (Ct) values were corrected by the median difference in all samples from Actb. All samples were then normalized by the difference from the median Ct of the three corrected control gene Ct levels in each sample, with the value converted to a nominal copy number per cell by assuming 2500 Actb mRNA molecules per cell and an amplification efficiency of 93% for all reactions. PCR results from DCs exposed to combinations of cytokines were normalized to values from untreated cells and log 2 transformed prior further statistical analysis. To get a picture of overall induction of those genes assayed, we summarized the log transformed expression levels on the most right column of **Figure 7**. Primers for genes can be found in the Table S3 in Supplementary Material.

T-CELL ACTIVATION ASSAY

PBMCs were exposed to inactivated native measles virus for 4 days. From these samples, CD3 cells were isolated by negative selection using the Pan T-Cell Isolation Kit II (Miltenyi) and stained with CFSE (Invitrogen). Those cells were then co-cultured with cytokine pretreated DCs which were also pulsed with inactivated native measles virus. T-cell proliferation was measured by the reduction of CFSE intensity of cells.

IN VIVO EXPERIMENTATION

Animal studies were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Mount Sinai School of Medicine. Animals were pretreated with murine 3.5 mg/kg BW IFNβ (PBL InterferonSource), murine 1.3 mg/kg BW TNFα (Peprotech), and 0.5 mg/kg IL1β (Peprotech) 6 and 3 h prior infection with the influenza A strain PR8 in an inhalation chamber.

STATISTICAL ANALYSIS

Micro array analysis was performed with the Partek Pro software 1-way ANOVA was calculated by using Method of Moments (14). Genes were compared by asymptotic unpaired *t*-test comparisons followed by a Benjamini–Hochberg multiple testing correction. All other data was analyzed with R. Maturation marker expression, apoptosis induction, and infectivity levels were first analyzed with ANOVA, followed with pairwise comparisons using the Tukey's "Honest Significant Difference" method. PCR of the gene expression after combinatorial treatment was also analyzed with ANOVA followed Tukey's "Honest Significant Difference" method and an additional Bonferroni multiple testing correction for the summarized data. Bead uptake data were analyzed with a pairwise Wilcoxon Rank Sum Test with the Bonferroni method for multiple testing correction. Survival of mice was analyzed with a Mantel–Haenszel test for survival analysis. Data as well as the R analysis can de downloaded from the supplementary data.

RESULTS

EXPOSURE TO PARACRINE SIGNALING FROM INFECTED DCs INDUCES GENE EXPRESSION PROFILE DISTINCT FROM THAT CAUSED BY IFNβ ALONE

The effects of paracrine signaling from infected DCs on naive DCs, transforming them to what we have previously referred to as anti-viral activated DCs [AVDC, Ref. (6)], were studied using microarrays (**Figure 1**). To compare the effects of paracrine signaling to the effects of a single cytokine treatment, we exposed naive DCs either to paracrine signaling from NDV infected cells (which generates AVDCs) or to IFNβ at a concentration found in the supernatants of NDV infected DCs. RNA samples from naive cells (CTRL) and NDV infected cells (NDV) were also assayed.

A PCA on the samples was performed in order to test how much individual samples are similar to the biological replicates within a group and how the different groups relate to each other. The PCA showed that cells exposed to paracrine signaling from infected DCs had a different overall expression profile than cells exposed to IFNβ alone (**Figure 1A**). Top genes for vectors were CXCL11, ISG20, ISG20, IDO1, IFI27, IFITM1, IFIT2, OASL, and CXCL9 for principal component (PC) 1; IFIT2, CXCL11, CCDC88A, NEXN, MALAT1, TNFSF10, P2RY12, SAMD9L, SMCHD1, and NEXN for PC2 PPBP, MMP1, ADAM12, IRG1, AKAP12, SLC28A3, DNAJC6, FABP4, ITGA9, and FABP4 for PC3. ANOVA (FDR $p < 0.05$) and

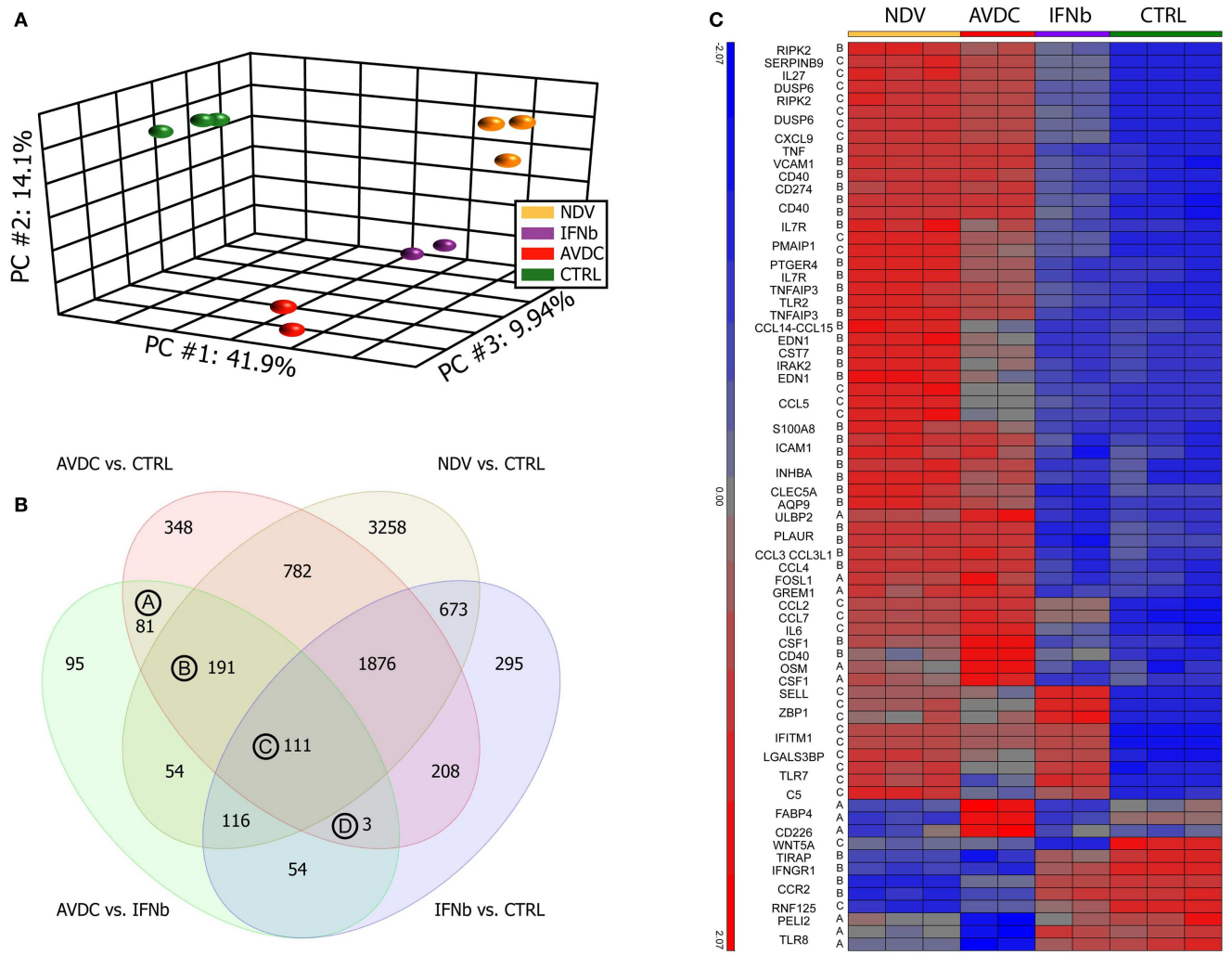

FIGURE 1 | Paracrine signaling from virus-infected DCs cells induce a different gene expression pattern compared to cells exposed to IFNβ alone. Microarray profiles were obtained from human monocyte-derived DCs exposed to paracrine signaling from NDV infected DCs or to IFNβ alone. Sample groups: NDV: DCs infected with NDV; IFNβ: DCs exposed to IFNβ alone; AVDCs: DCs exposed to paracrine signaling from NDV infected cells in a trans-well system; CTRL: naive unexposed and uninfected DCs. **(A)** Principal component analysis of samples on all genes represented on the microarray. First three principal components showing 41.9% (PC1), 14.1% (PC2), and 9.94% (PC3) of the overall change in gene expression in the data set. **(B)** Venn diagram comparing genes which differed (twofold, FDR $p < 0.05$) between each pair of conditions. **(C)** Genes which are differed between AVDC vs. CTRL and AVDC vs. IFNβ and have a gene ontology association with anti-viral immune processes.

twofold change threshold relative to control cells identified 7088 genes altered by NDV infection, 3600 genes altered by paracrine signaling (AVDC), and 3336 genes changed by IFNβ alone.

The number of genes differentially expressed between each pair of the four groups studied is indicated in **Figure 1B**. A comparison between cells exposed to paracrine signaling and IFNβ alone showed 705 differentially expressed genes. From those 705 transcripts, which showed a significant change between exposure to paracrine signaling and single cytokine IFNβ treatment, 81 were significantly altered by the paracrine signaling but did not show significant induction by NDV infection or IFNβ treatment when compared to control [Group (A) in **Figure 1B**], 191 genes were significantly induced by paracrine signaling and NDV infection but not IFNβ treatment when compared to control [Group (B) in **Figure 1B**] and 111 genes were significantly induced by paracrine signaling, NDV infection, and IFNβ treatment when compared to control [Group (C) in **Figure 1B**]. Three genes were changed by paracrine signaling and IFNβ treatment when compared to control but still differed significantly when compared between exposure to paracrine signaling and IFNβ treatment. Heat maps of all genes in groups A, B, C, and D can be seen in the supplementary material (Figure S2 in Supplementary Material). Fifty genes from the list of 389 transcripts being significantly different when exposed to paracrine signaling vs. IFNβ alone, as well as when exposed to paracrine signaling and naive cells could be linked to anti-viral immunity (**Figure 1C**). Among those, 50 genes were regulators

of inflammation and immune response including *VCAM-1* (17), *AQP9* (18), *RIPK2* (19), *IRAK2* (20), *CCL3L1* (21); cytokines like *IL6*, *OSM* (22); genes linked to anti-viral immunity *CCL3L3* (23), *CSF1* (24), *CD274* (25), *CD40* (26), *IL7R* (27); immune cell activation *CLEC5A* (28), *EDN* (29), *CST7* (30), and also a suppressor of apoptosis *PTGER4* (31).

BYSTANDER DCs ARE EXPOSED TO A COMPLEX CYTOKINE ENVIRONMENT

To identify cytokines and chemokines induced during NDV infection, to which uninfected bystander DC cells are exposed, we analyzed the 7088 transcripts induced by NDV. Seventy-eighty transcripts could be associated to the gene ontology terms cytokine activity or chemokine activity (**Figure 2A**). We further narrowed this list by setting an expression threshold of 6.5 based on the intersection of the two populations of expressed and non-expressed genes (**Figure 2B**) and identifying which cytokines/chemokines could be associated with receptor genes also expressed in DCs. This analysis linked CCL4 to CCR5 (32, 33), CCL3 to CCR5 (34), CCL3 to CCR1 (35), CCL2 to CCR2 (36, 37), CCL7 to CCR5 (33), CCL7 to CCR3 (38), CCL8 to CCR2 (39), CXCL10 to CCR3 (40), CXCL9 to CCR3 (40), CXCL11 to CCR3 (40), CCL15 to CCR3 (41), CCL15 to CCR1 (41), CCL5 to CCR3 (36), CCL5 to CCR5 (42, 43), CXCL12 to CXCR4 (44), CXCL3 to CXCR2 (36), CXCL5 to CXCR2 (45), CXCL1 to CXCR2 (46), IL6 to IL6R (47), IL1a to IL1R1 (48), IL1β to IL1R2 (49), IL1β to IL1R1 (50), TNF to TNFRSF1B (51), TNF to FAS (52), TNF to TNFRSF1A (53), IL15 to IL15RA (54), IL7 to IL7R (55), IL7 to IL2RG (56), IFNβ to IFNAR1 (57), IFNβ to IFNAR2 (58), IFNW1 to IFNAR1 (59), IFNA2 to IFNAR (60), IFNE to IFNAR1 (61), TNFSF10 to TNFRS10B (62), CCL19 to CCR7 (63), TNFSF15 to TNFRSF6B (64), IL28A to IL10RB (65), IL29 to IL10RB (65), IL12A to IL12RB1 (66), IL12A to IL12RB1 (67), and CSF1 to CSF1R (68) (**Figure 2C**).

The level of expression of cytokines and chemokines identified by this bioinformatics analysis was measured in an 18-h time course experiment (1) in supernatant from DCs infected by NDV, (2) in the supernatant associated with AVDCs in trans-well experiments, and (3) in supernatant of cells exposed to IFNβ alone by ELISA (**Figure 3A**) or in cellular mRNA by real-time PCR (**Figure 3B**). Cytokines which did not exhibit detectable expression by ELISA or PCR (not shown) were excluded for further screening. This led to the selection of the following 20 cytokines and chemokines for further study that were induced in NDV infected DCs: TNFα, CCL3, IL8, CXCL10, CCL5, IL6, IFNα CXCL12, IFNALPHA16, IL12a, IL18, IL1RA, IL28, IL29, TNFSF15, TNFSF4, TNFSF10, IL1α, IL1β, and IFNβ.

IDENTIFICATION OF INDIVIDUAL CYTOKINES CONTRIBUTING TO COMBINATORIAL EFFECTS

We next studied the combinatorial effects of the 20 cytokines identified above on the induction of maturation marker expression in naïve DCs. Because studying all combinations of 20 cytokines was impractical, we identified combinatorial cytokine candidates by comparing the effect of all 20 cytokines on naïve DCs to the effects of all possible 19-cytokine combinations lacking one of the cytokines. These experiments used the maximum concentration measured by ELISA or, for cytokines measured by PCR, the concentration was estimated from transcript levels by comparing the PCR and ELISA levels of IFNβ. Many cytokines peaked at about 10 h during the 18 h time course. Therefore, we expose DCs to the cytokine mixtures for 8 h to best approximate the conditions of the paracrine signaling during viral infection. In this experiment, we used cells from three different donors for biological replicates, which resulted in a high variance of marker expression between donors. To adjust for differences between individuals, we normalized data to the overall median values.

The absence of TNFα, IL18, IL28, and IFNalpha16 reduced the expression of CD86, when compared to the exposure to all 20 cytokines (**Figure 4**). The absence of IFNa2, IL18, IL1α, TNFSF15, IL1β, and IFNβ reduced the expression of HLA-DR (**Figure 4**). We studied nine cytokines (IFNα, IFNALPHA16, IFNβ, IL1α, IL1β, IL18, IL28, TNFα, TNFSF15) in similar leave one cytokine out experiments as well as single cytokine exposure studies. These nine cytokines gave the same responses as the original 20, indicating that the 11 cytokines excluded from further study are not major contributors to maturation marker induction during paracrine signaling (**Figure 5**). The cytokine minus one studies with the remaining nine cytokines suggested the importance of IFNβ, for CD86 upregulation and IFNβ, IL28, and TNFSF15 for HLA-DR upregulation. When DCs were exposed to individual cytokines, IFNβ and IL1β induced CD86 and IFNβ and TNFα induced HLA-DR. Therefore five cytokines (IFNβ, IL28, TNFSF15, TNFα, IL1β) were selected for further study.

TNFα, IFNβ, AND IL1β INDUCE A PARACRINE ACTIVATED ANTI-VIRAL STATE

We studied the effects of combinations of the five cytokines on maturation marker expression, viral resistance, and phagocytic activity. For the maturation marker expression studies, human DCs were exposed to combinations of the five cytokines for 8 h and the levels of CD86 and HLA-DR were measured by flow cytometry. IFNb alone increases the expression of both markers. The additional increases observed with all combinations of cytokines did not achieve statistical significance in comparison to IFNb alone with tight control for family wise error. IL28 did not cause any trend toward an increase in maturation marker expression (**Figure 6A**). To improve statistical power, we performed maturation marker induction experiments using four cytokines IFNβ, TNFSF15, TNFα, and IL1b. Here, the combinations of IFNβ with either TNFα or IL1b showed a significantly higher induction of CD86 when compared to the effects of IFNβ alone (**Figure 6B**). The combination of IFNβ with IL1b showed a significantly higher HLA-DR induction when compared to IFNb alone (**Figure 6C**).

As one of the most important features observed in paracrine activated AVDCs is resistance to viral infection (6), we pretreated DCs with combinations of the five cytokines for 8 h and subsequently infected them with an RFP expressing NDV for 8 h. Infectivity was measured by using flow cytometry to quantify RFP expression. Because infectivity by RFP-NDV is so sensitive to IFNβ, its concentration was reduced for this study. Still, due to the effect of the RFP insertion which makes the virus less viable and more susceptible to the effects of IFNβ, changes observed with cytokine

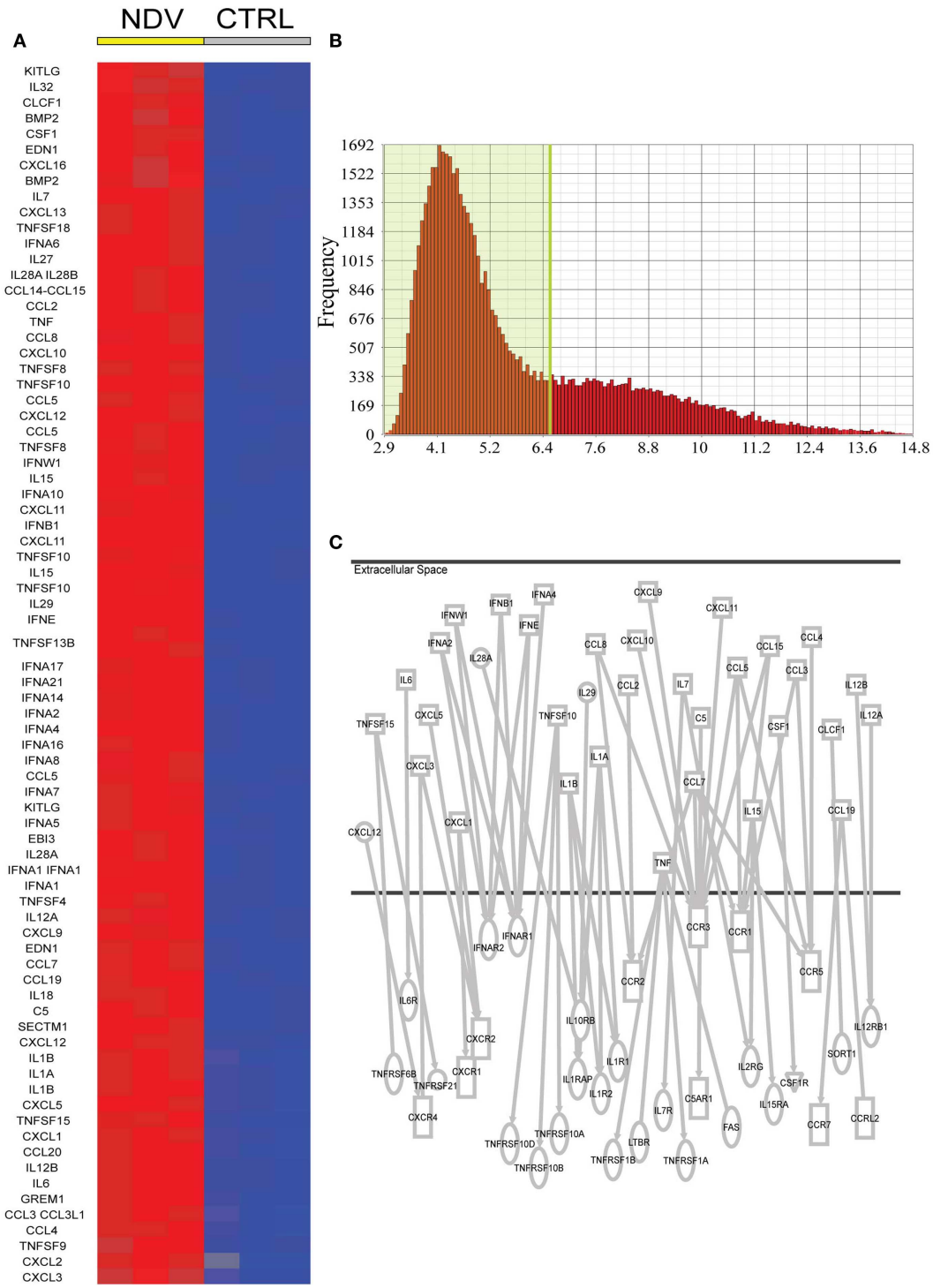

FIGURE 2 | Candidate NDV induced cytokines in DCs for a paracrine effect on DCs. **(A)** Cytokines and chemokines induced by NDV (twofold FDR $p < 0.05$) compared to naive DCs. **(B)** Expression level of all genes expressed in naive DCs. A cutoff of expression above 6.5 was used to identify potentially expressed cytokine and chemokine receptors. **(C)** Ingenuity analysis linking induced cytokines/chemokines to receptor genes also expressed in DCs.

combinations were statistically not significant when compared to single cytokine IFNβ exposure. The combination of IFNβ and IL1β showed the largest reduction of infection (**Figure 6D**).

Another feature of AVDCs is the heightened phagocytic activity (10). Therefore, we tested fluorescent bead phagocytosis following exposure to combinations of IFNβ, TNFSF15, TNFα, and IL1β.

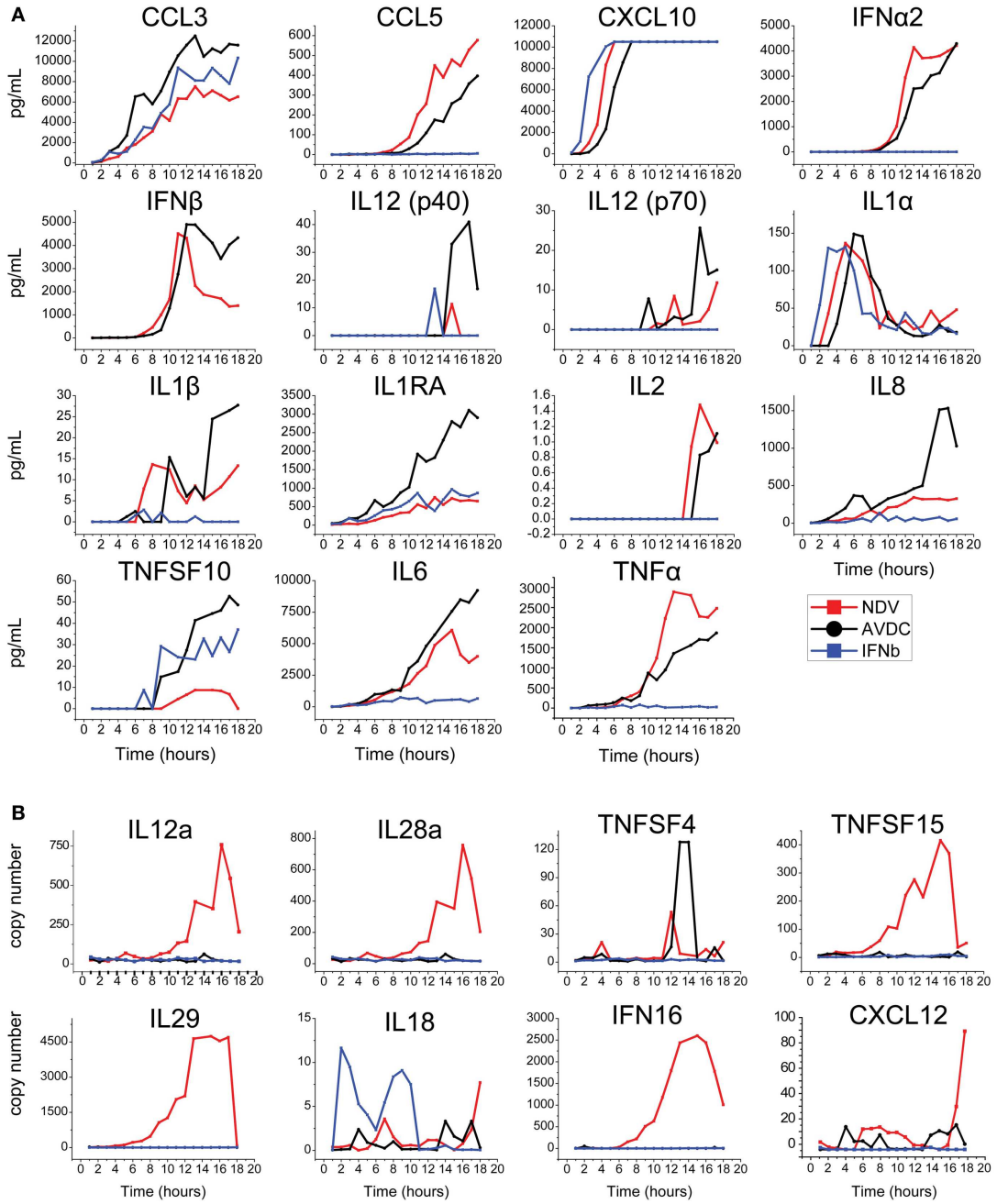

FIGURE 3 | Time course of expression of cytokines/chemokines in NDV infected DCs, AVDC, and IFNβ exposed DCs determined by (A) multiplex ELISA or IFNβ ELISA or (B) real-time PCR over an 18 h period. NDV (red line) indicates measurements from supernatants (ELISA) or cells (PCR) which were infected with NDV at an MOI of 1. AVDC (black line) indicates measurements from supernatants from infected and co-cultured naïve DCs at a 1:1 ratio (ELISA) or naïve DCs which were co-cultured with infected cells (PCR). IFNβ (blue line) indicates measurements from supernatants (ELISA) or cells (PCR) from naïve DCs exposed to IFNβ alone.

To improve statistical power and in view of the lack of effect on maturation markers, IL28 was excluded from this study. Cells were pretreated with cytokine combinations for 8 h, and then co-cultured with fluorescent beads for 4 h. The number of beads in each cell was then counted using imaging flow cytometry. The highest rates of phagocytosis were seen with all four cytokines exposed together and with exposure to IFNβ with either IL1β or TNFSF15 (**Figure 6E**).

We also looked at the capacity of combinations of the five cytokines to regulate 16 immune related genes that had been identified as preferentially induced by paracrine signaling in comparison with IFNβ exposure alone (see **Figure 1C**). Here, we exposed

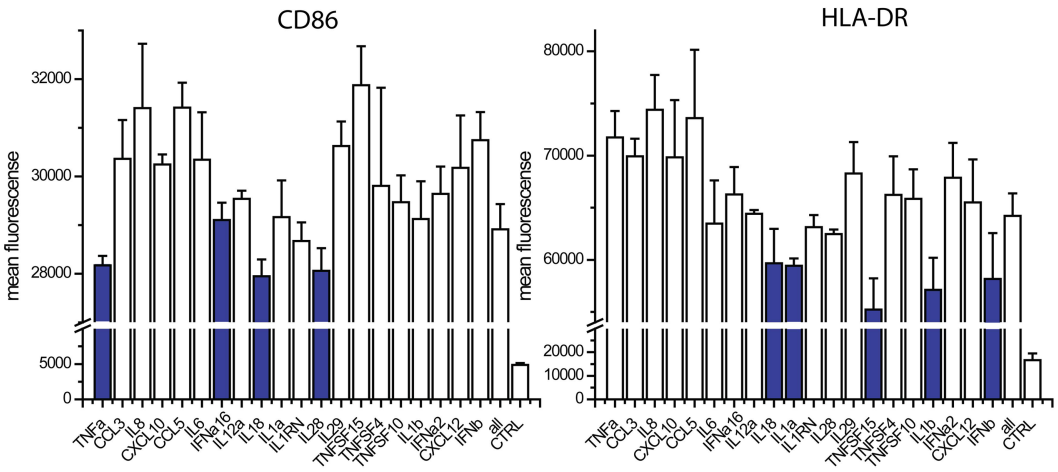

FIGURE 4 | Contribution of individual cytokines in 20 cytokine paracrine signal. Maturation marker expression of DCs after 8 h to exposure to all 20 cytokines (all) or the leave-one-out combinations of 19 cytokines. Note that a reduction in expression with a cytokine absent indicates that the cytokine may contribute to induction.

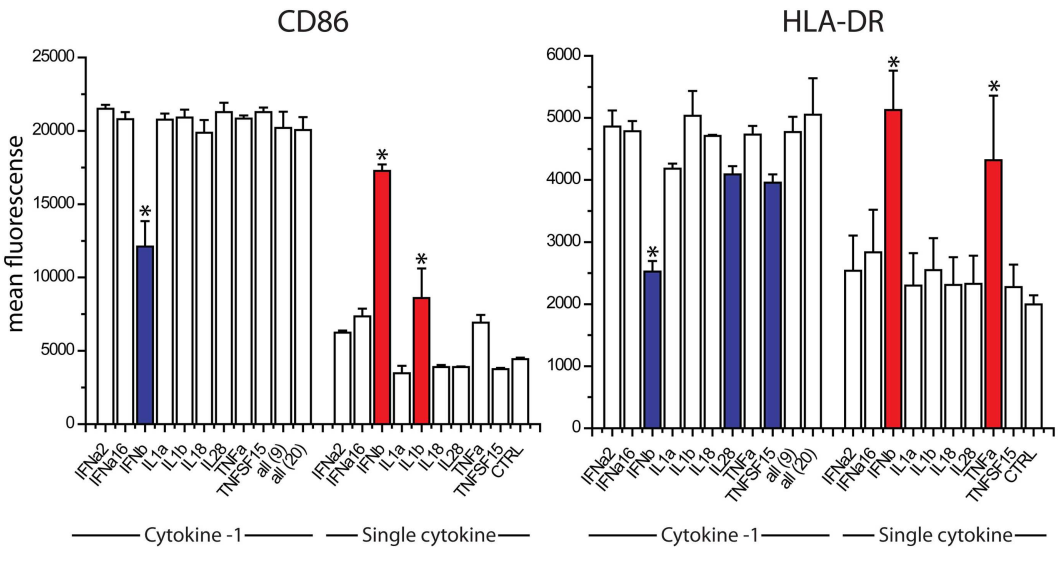

FIGURE 5 | Contribution of individual cytokines in nine cytokine paracrine signal. Maturation marker expression of DCs after 8 h to exposure to all nine cytokines, all leave-one-out combination of eight cytokines and each individual cytokine treatment (Single cytokine) ($*p \leq 0.002$ to cells exposed to all nine cytokines in Cytokine-1 treatment and to untreated cells in the single cytokine treatments).

DCs for 8 h to all possible combinations of IFNβ, IL28, TNFSF15, TNFα, IL1β, mRNA, and performed qPCR. Gene expression is plotted on a heatmap (**Figure 7**). The genes assayed were *VCAM-1*, *AQP9*, *RIPK2*, *IRAK2*, *CCL3L1*, *IL6*, *OSM*, *CCL3L3*, *CSF1*, *CD274*, *CD40*, *IL7R*, *CLEC5A*, *EDN*, *CST7*, and *PTGER4*). Nine of these genes showed the highest induction when treated with the triple combination of IFNβ, TNFα, and IL1β (**Figure 7**). The genes showing combinatorial cytokine preferences are associated with antiviral immunity (*CCL3L3*, *CSF1*, *IL7R*), regulation of inflammation (*AQP9*, *IRAK2*, *RIPK2*), and immune cell activation (*CST7*, *EDN*, cytokine *OSM*). The induction of IL6 was also high with the triple cytokine treatment. Overall, these experiments demonstrate that IFNβ, TNFα, and IL1β acting together are the principal drivers of the paracrine induced anti-viral state in DCs.

We next performed concentration response studies to determine if the combinatorial effects of the cytokines were synergistic. At lower concentrations, all three cytokines together produced the highest levels of both CD86 expression although at higher concentrations, equivalent levels could be achieved with the combination of TNFα and IFNβ alone (**Figure 8**). For HLA-DR expression, the combination of all three cytokines at lower concentrations produced the highest levels, although at higher concentrations TNFα and IL1β induced similar levels. For both maturation markers, the effects of combinatorial

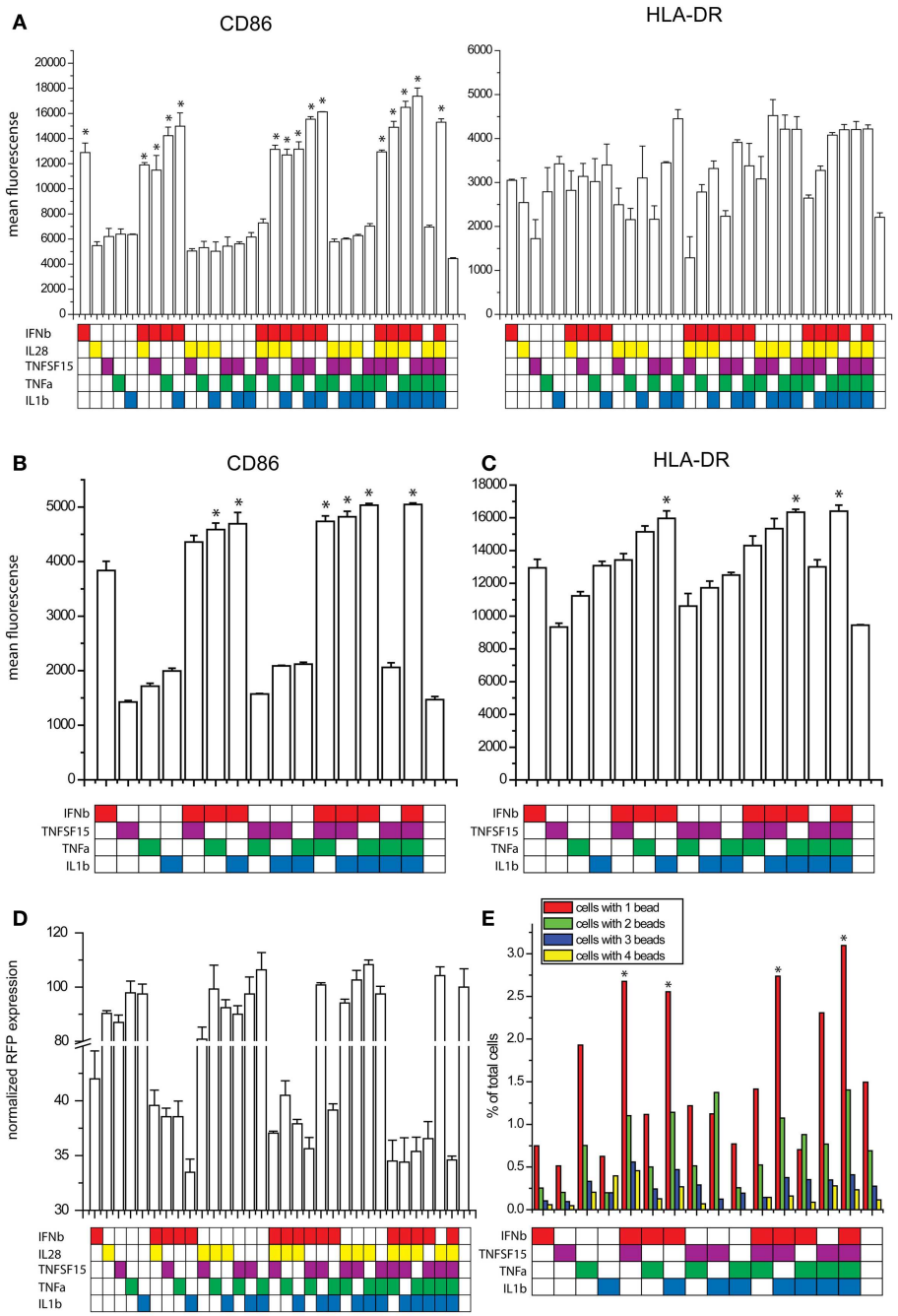

FIGURE 6 | Effects of combinatorial cytokine treatment. **(A)** Maturation marker expression after exposure to combinations of IFNβ, IL28, TNFSF15, TNFα and IL1β, measured by flow cytometry (*$p \leq 0.05$ compared to untreated cells). **(B)** CD86 expression after exposure to combinations of IFNβ, TNFSF15, TNFα and IL1β, measured by flow cytometry (*$p \leq 0.05$ compared to cells treated with IFNβ alone). **(C)** HLA-DR expression after exposure to combinations of IFNβ, TNFSF15, TNFα and IL1β, measured by flow cytometry (*$p \leq 0.05$ compared to cells treated with IFNβ alone). **(D)** Infectivity of NDV after cytokine combination pretreatment for 8 h in DCs measured by RFP expression. **(E)** Phagocytosis assay measured by bead uptake by imaging flow cytometry (*$p \leq 0.05$ compared to untreated cells).

exposure dramatically exceeded the effects of any individual cytokine. Statistical values for pairwise comparisons within each dilution step are shown in Tables S1 and S2 in Supplementary Material. We also investigated the effect of combinations of various combinations of the two cytokines IFNβ and TNFα, which showed a synergistic induction with CD86 (Figure S3 in Supplementary Material). These results support the view that the effects of combinatorial exposure produce a qualitatively different cellular effect than do the individual cytokines.

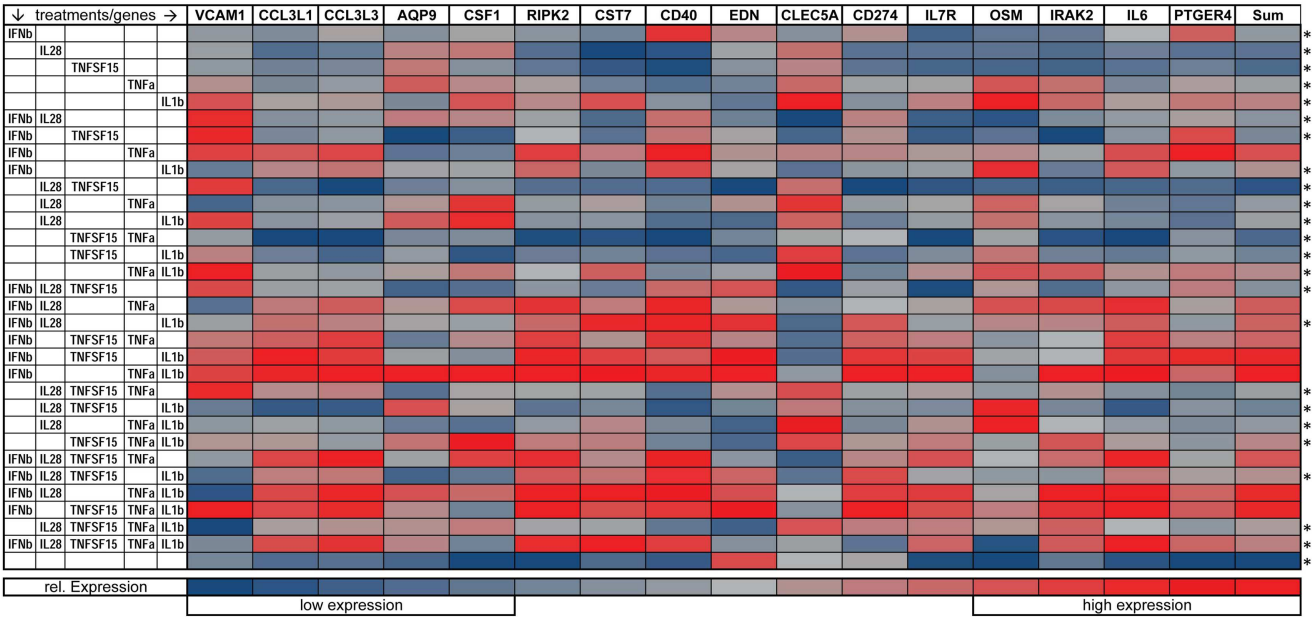

FIGURE 7 | Gene expression of immune relevant genes after combinatorial IFNβ, IL28, TNFSF15, TNFα, and IL1β treatment. Heat map is normalized to the lowest (blue) and highest (red) expression level for each individual gene (*$p \leq 0.05$ compared to cells exposed to the triple combination of TNFα, IFNβ, and IL1b).

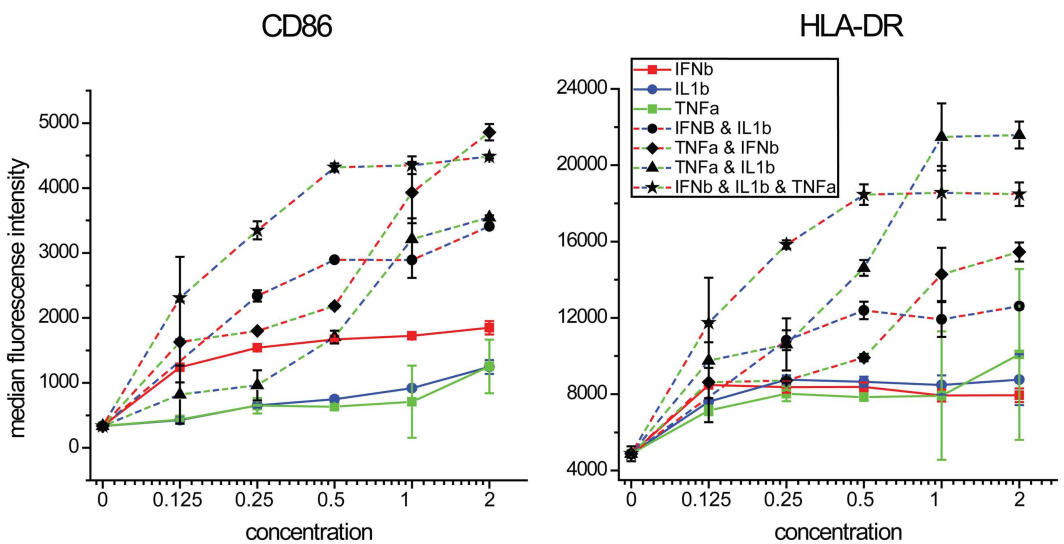

FIGURE 8 | Maturation marker expression by combinations of IFNβ, TNFα, and IL1β. DCs were exposed to combinations of three cytokines at a range of concentrations relative to that found in supernatants of NDV infected DCs.

COMBINATORIAL EFFECTS OF TNFα, IL1β, AND IFNβ ON PATHOGENIC VIRUSES

The studies described above rely on NDV, which is not pathogenic in humans and does not express immune antagonists having activity in human cells. We were interested in studying the effects of combination cytokine exposure on a human pathogen and studied the recent pandemic Influenza A virus Cal/09. We pretreated DCs with all combinations of IFNβ, TNFα, and IL1β for 8 h and subsequently infected them with influenza A/California/7/2009 and measured infectivity, maturation marker induction as well as induction of apoptosis. The triple combination of all three cytokines as well as the dual cytokine mixture of IL1β and IFNβ significantly increased the suppression of infectivity when compared to IFNβ pretreatment alone (**Figure 9A**). The triple combination also caused the highest induction of CD86, HLA-ABC, and HLA-DR in virus NP-expressing cells (**Figures 9B–D**).

Apoptosis was assayed in the same samples using imaging flow cytometry to measure nuclear fragmentation (**Figures 9F,G**). Interestingly, pretreatment with single cytokines did not reduce influenza-induced apoptosis, whereas the triple combination as well as the dual cytokine combinations with IFNβ could reduce cell death (**Figure 9E**). These data suggests that the triple combination of cytokines improves DC survival, resistance to infectivity, and increases costimulatory marker expression for T-cell activation.

COMBINATORIAL EFFECT OF TNFα, IL1β, AND IFNβ ON INDUCTION OF VIRUS SPECIFIC T-CELL RESPONSE

In order to see if the induction of the costimulatory markers by the triple combination of IFNβ, TNFα, and IL1β has an effect on T-cell activation, we studied the induction of measles specific T-cell proliferation after co-culture with cytokine pretreated and measles primed T-cells. We exposed the CD14 depleted PBMCs from the same donors which were used for DC generation to measles vaccine and harvested T-cell 5 days later. Those T-cells were co-cultured for 3 days with measles primed DCs exposed to combinations of IFNβ, TNFα and IL1β. Proliferation was measured by the dilution of the membrane bound dye CFSE by flow cytometry. The triple combination of IFNβ, TNFα, and IL1β significantly increased cell proliferation compared to non-pretreated DCs (**Figure 10**). These results indicate that the costimulatory marker upregulation also affects activation of the adaptive immune system.

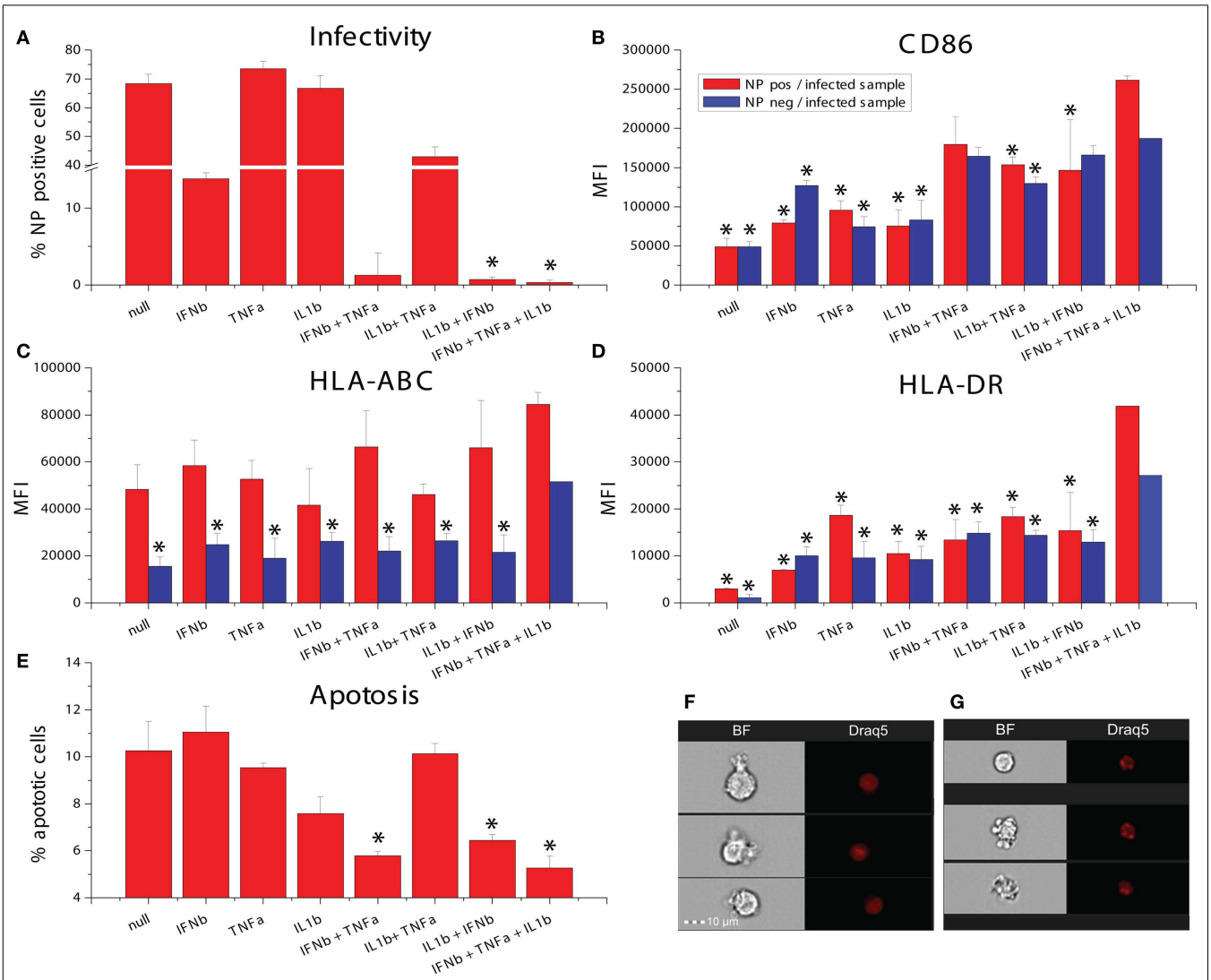

FIGURE 9 | Effects of combinatorial of IFNβ, TNFα, and IL1β treatment on DCs infected with the pandemic influenza A Cal/09 strain. **(A)** Infectivity assayed by NP expression (*$p \leq 0.05$ compared to cells exposed to IFNβ). **(B)** Expression of the maturation marker CD86. **(C)** Expression of the maturation marker HLA-ABC. **(D)** Expression of the maturation marker HLA-DR (*$p \leq 0.05$ compared to cells exposed to the triple combination of TNFα, IFNβ, and IL1b). **(E)** Apoptosis assayed by assessing nuclear fragmentation and cell granularity by imaging flow cytometry (*$p \leq 0.05$ compared to untreated cells). **(F)** Sample images of non-apoptotic cells. **(G)** Sample images of apoptotic cells.

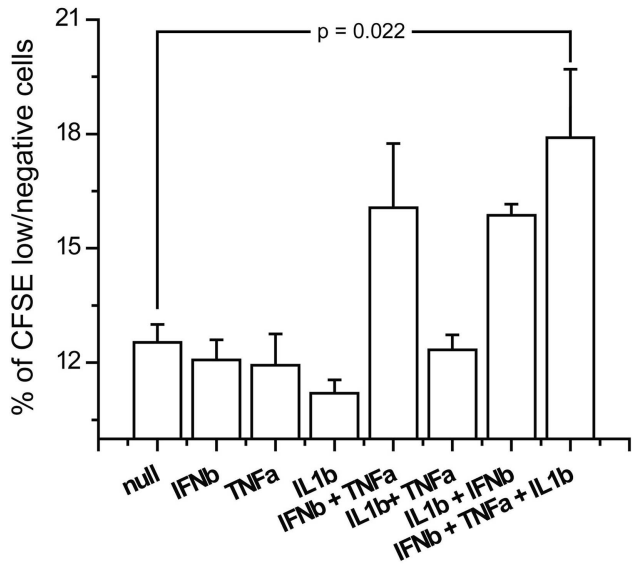

FIGURE 10 | Antigen specific proliferation of T-cells exposed to cytokine pretreated DCs. Measles antilogous specific T-cells were co-cultured with cytokine pretreated DCs which were also pulsed with measles vaccine. Proliferation was measured by the dilution of CFSE.

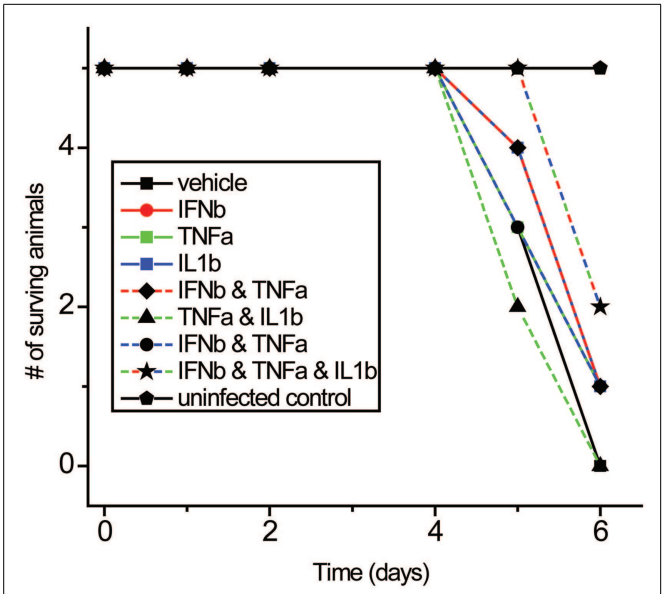

FIGURE 11 | Effects of IFNβ, TNFα, and IL1β combinations on mortality with PR8 virus *in vivo*. Mice received cytokine injection i.p. 6 h before and 6 h after infection with PR8 virus.

COMBINATORIAL EFFECT OF TNFα, IL1β, AND IFNβ ON INFLUENZA MORBIDITY *IN VIVO*

We next studied the effects of IFNβ, TNFα, and IL1β on influenza virus pathogenicity *in vivo* using a well-characterized aerosolized-virus mouse infection model (69). Cytokines were injected intraperitoneally both 3 h before and after inhalation infection with PR8 virus. While the differences were modest, the triple combination was found to improve survival times significantly compared to control (p: 0.0456) following PR8 infection in mice (**Figure 11**).

DISCUSSION

In this study, we show that IFNβ, TNFα, and IL1β are secreted by virus-infected DCs and act combinatorially to alter the anti-viral response state of uninfected DCs. This combination is responsible for maturation marker upregulation in naive as well as in infected cells, reduction of virus induced apoptosis, heightened phagocytic activity, specific autologous T-cell activation, and resistance to viral infection *in vitro* as well as in *in vivo*.

The importance of cellular micro-environments in dictating immune cell responses is supported by the report that the inflammatory state of macrophages can be reprogramed by exposure to an anti- or pro-inflammatory stimuli (70). Another report has suggested that the initial exposure to a cytokine signal determines and fixes the final state of the macrophage (71). These reports, as well as the finding that the combination of IL-4, IL-10, and TGFb skew the development of myeloid cells into M2 macrophages, support the importance of combinatorial cytokine signals in immune regulation (72). DCs themselves are differentiated into different lineages by exposure to different cytokines including GMCSF and Flt3 (73).

Since the discovery of type I interferon, paracrine cytokine signaling has been recognized as a crucial component in orchestrating the immune responses to virus infection. Recent studies have begun to reveal the importance of combinatorial extracellular stimuli in directing the responses of immune cells. For example, when DCs are exposed to lipopolysaccharide in the context of apoptotic cells, they induce TH17 cells, a response that is not achieved by either stimulus alone (74). Retinoic acid acts alone on T-cells to induce Treg cells. However, retinoic acid combined with IL-15 causes DCs to skew the T-cell polarization toward TH17 cells (6). TLR7/8 ligand combined with either TLR3 or TLR4 ligands synergistically increases IFNβ and IFNλ1 expression in DCs (75). SCF and IL-2 have a synergistic effect on the proliferation NK cells (7). TNFα and IFNγ act together on smooth airway cells to enhance CXCL-10 expression (8). IL17 together with TNFα or IL1β induces MCP-1 and MIP-2 in murine mesangial cells (9). These combinatorial effects are likely to prove clinically relevant, for example, by contributing to individual differences in the response to cytokine treatment (76). While beyond the scope of the present investigation, the role of relative timing of combinatorial cytokine signals is another important area for further study. We have also not addressed the potential combinatorial role of alarmins, which can work in concert with cytokines to induce different cell states (77).

To our knowledge, this is the first report of IFNβ, TNFα, and IL1β working in concert to alter the response state of any immune cell. Previous studies have implicated pairs of this triad in influencing immune responses. The combination of IL-1β and IFNβ has been reported to promote immune control of West Nile virus infection in the CNS (78). TNFα and IFNβ have also been found to affect macrophages and fibroblasts in reducing the infectivity of poxviruses (79, 80).

Several of the transcripts that are preferentially induced in DCs by the exposure to all three cytokines have been found to serve important roles in inflammation and immunity: CCL3L3 suppresses HIV proliferation (23); AQP9 is a marker for inflammation (81); CSF1 is a biomarker for respiratory syncytial virus infection (24); RIPK2 knockout in mice causes hyper-susceptibility to infection with influenza A virus (82); EDN possesses anti-viral activity against single stranded RNA viruses like respiratory syncytial virus, Hepatitis and HIV (83); IL7R expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ T reg cells (84); OSM is a pro-inflammatory cytokine (22); and IRAK2 is needed to sustain cytokine production during prolonged activation of the TLR signaling pathway (85). When maturation marker induction was studied by cytokine induction alone, we found that CD86 is most strongly driven by IFNβ with synergistic effects of TNFα or IL1β. HLA-DR was little changed by individual cytokines but was strongly induced by TNFα and IL1β together. The gene VCAM-1 was most induced by the TNFα and IL1β together and PTGER4 was most induced by IFNβ and TNFα. While the overall DC cell state observed requires all three cytokines, the differences in the cytokines most important for various components of these DC responses provides the basis for future studies to dissect the underlying signaling and transcriptional mechanisms involved in these combinatorial effects.

Surprisingly, the triple combination of IFNβ, TNFα, and IL1β reduced influenza-induced cell death in infected DCs. This is interesting as IFNβ is known to be an inducer of apoptosis in DCs (86), and indicates how the effects of one cytokine may be very different depending on which other cytokines are stimulating a cell. Maturation marker induction as well as cell survival are important for the activation of the adaptive immune system. The observation of a heightened proliferation of virus specific T-cells when exposed to DCs pretreated with the triple combination supports this view. The modestly increased survival of mice to PR8 infection when treated with the three cytokines suggests the combinatorial coding of cell responses has significance *in vivo*.

The large number of cytokines secreted by infected DCs is remarkable. We identify combinatorial effects involving only three of these secreted factors on DCs. It is probable that combinatorial signaling of different cytokine mixtures influences the activation state of other immune cells and that other immune cells also serve as the source of complex cytokine signals. The specific activation state of any immune cell can depend on both the cytokine mixture present and their concentration (see **Figure 8**). Thus the immune system can potentially generate many distinct micro-environments that shape the local activation state of various immune cells. This provides the potential for a dynamic and spatially distributed complexity of the set point of the immune system that could be crucial in organizing the local and system responses to infection, neoplasia, and injury. Unraveling this combinatorial code may have benefits in guiding combination immunotherapy for autoimmune diseases, for chronic infections and for other immune system influenced diseases as well as for personalizing interventions in relationship to individual variation in background cytokine expression.

ACKNOWLEDGMENT

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00073/abstract

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Global inhibition of DC priming capacity in the spleen of self-antigen vaccinated mice requires IL-10

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Dendritic cells (DC) in the spleen are highly activated following intravenous vaccination with a foreign-antigen, promoting expansion of effector T cells, but remain phenotypically and functionally immature after vaccination with a self-antigen. Up-regulation or suppression of expression of a cohort of pancreatic enzymes 24–72 h post-vaccination can be used as a biomarker of stimulatory versus tolerogenic DC, respectively. Here we show, using MUC1 transgenic mice and a vaccine based on the MUC1 peptide, which these mice perceive as a self-antigen, that the difference in enzyme expression that predicts whether DC will promote immune response or immune tolerance is seen as early as 4–8 h following vaccination. We also identify early production of IL-10 as a predominant factor that both correlates with this early-time point and controls DC function. Pre-treating mice with an antibody against the IL-10 receptor prior to vaccination results in DC that up-regulate CD40, CD80, and CD86 and promote stronger IFNγ+ T cell responses. This study suggests that transient inhibition of IL-10 prior to vaccination could improve responses to cancer vaccines that utilize self-tumor antigens.

Keywords: IL-10, dendritic cells, cancer vaccine, MUC1, T cell response

INTRODUCTION

The impact of IL-10 on the cells of the immune system is well studied and varied. Originally identified as cytokine synthesis inhibitory factor, IL-10 can play a role in the development and maturation of almost all immune cells (1, 2). Signaling through the IL-10 receptor (IL-10R) occurs through a STAT3 intermediate and is known to induce SOCS-3 expression, to suppress IFN signaling by blocking STAT1 phosphorylation, and to inhibit NF-κB signaling by preventing its nuclear translocation as well as inhibiting its binding to DNA (2, 3). In dendritic cells (DC), known for being the most important professional antigen presenting cells, IL-10 can reduce expression of MHC Class II and the costimulatory molecules CD80/86 and CD40, as well as reduce IL-12 secretion (3–6). This is true even for DC previously activated with IFNγ. IL-10 can also prevent monocyte differentiation into DC (2).

IL-10 has a profound effect on T cells as well. For example, reduced IL-12 production by DC affected by IL-10 antagonizes the development of T helper type 1 (Th1) responses while reduced MHC II levels on DC result in presentation of low density antigen that preferentially stimulates differentiation of regulatory CD4 T cells (7, 8). IL-10 can also act directly on T cells to inhibit synthesis of cytokines like IL-2 and IFNγ in CD4 T cells or to inhibit their proliferation (3). The effect of IL-10 on CD8 T cells is less clear although some studies have shown that IL-10 can favor activation of CD8 T cells (9–11).

Recently, our group implicated IL-10 in controlling in part the function of DCs post-vaccination with antigens derived from self-proteins. Using the MUC1 transgenic (MUC1.Tg) mouse model and a peptide derived from the extracellular domain of the tumor antigen MUC1, we showed that 24 h following vaccination, there is an IL-10 dependent suppression of DC activation that is detectable via suppression of expression of a newly discovered biomarker: a cohort of pancreatic enzymes. These enzymes, expressed in the spleen only by DC and represented by trypsin 1 and carboxypeptidase B1 (CPB1), are up-regulated post-vaccination with a foreign but not a self-antigen and identified a DC population that has higher MHC Class II, higher costimulatory molecule expression, and a higher T cell stimulatory capacity (12).

In this study, we present new evidence of an important role for IL-10 in the suppression of splenic DC following intravenous vaccination with a self-antigen. We show an early (4–8 h) up-regulation in IL-10 levels in spleens of self-antigen vaccinated mice that is not seen in mice that see that same antigen as foreign and coincides with the time when we also see differences in biomarker enzyme expression. Furthermore, DC in the spleens of self-antigen vaccinated mice have an increased sensitivity to IL-10. When the effect of IL-10 is blocked by pre-vaccination treatment of mice with an anti-IL-10R blocking antibody, there is a significant increase in the activation level and stimulatory capacity of DC at 24 h post-vaccination and a significant increase in CD4 T cell responses 7 days post-vaccination. These data implicate IL-10 in the regulation of antigen-specific immunity versus tolerance at a previously underappreciated early time post-vaccination, and suggest that manipulating its function at the time of vaccination might overcome tolerance and improve responses to cancer vaccines that utilize self-antigens.

MATERIALS AND METHODS

MICE

Human MUC1.Tg mice (13) on the C57Bl/6 background were a generous gift from Dr. Sandra Gendler (Mayo Clinic) and were bred and maintained in the University of Pittsburgh Animal

Facility. C57Bl/6 (WT) mice were purchased from The Jackson Laboratory. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

MUC1 VACCINATION

A 100-aa peptide containing five repeats of the MUC1 VNTR sequence HGVTSAPDTRPAPGSTAPPA, was synthesized as previously described (14) by the University of Pittsburgh Genomics and Proteomics Core Laboratories. For soluble peptide vaccinations, 100 μg of this 100mer peptide admixed with 50 μg polyinosinic–polycytidylic acid and poly-L-lysine (Poly-ICLC; Hiltonol) was brought up to 100 μL with PBS and injected via tail vein. For DC-based vaccinations, DC were prepared as previously described (15). Briefly, RBC lysed bone marrow cells were put into culture for 6 days in AIM-V supplemented with 10 ng/mL GM-CSF (Miltenyi), feeding once on day 3. On day 6, semi-adherent cells were collected by gentle agitation and put into culture overnight in AIM-V containing 33 μg/mL MUC1 100mer peptide and 25 μg/mL Poly-ICLC. The next day, mature DC were collected and resuspended in PBS at a final concentration of $0.5^{-1} \times 10^6$ cells/mL. One hundred microliters of this solution was then injected intravenously via tail vein.

IL-10R BLOCKADE

Where indicated, mice were given 250 μg of an antibody against the IL-10R (Bio X Cell, Clone 1B1.3A) or an isotype-matched control antibody (Bio X Cell, Clone HPRN), intraperitoneally. Twenty-four to forty-eight hours following treatment, mice were vaccinated as described in "MUC1 vaccination" above and analyzed as described.

QUANTITATIVE RT-PCR

RNA was extracted from whole spleen using TRIzol (Invitrogen) according to the manufacturer's protocol. Following extraction, cDNA was generated using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen). qPCR was performed using QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's protocol. Reactions were run on a StepOnePlus instrument (Applied Biosystems). The following primer pairs were used: trypsin 1 (forward: 5′ACTGTGGCTCTGCCCAGCTC3′; reverse: 5′AGCAGGTCTGGTTCAATGACTGT3′), CPB1 (forward: 5′GCCCTGGTGAAAGGTGCAGCAAAGG3′; reverse: 5′AG CCCAGTCGTCAGATCCCCCAGCA3′), IL-10 (forward: 5′CTTC CCAGTCGGCCAGAGCCA3′; reverse: 5′ CTCAGCCGCATCCTG AGGGTCT3′), and HPRT (forward: 5′TGAGCCATTGCTGAGGC GGCGA3′; reverse: 5′CGGCTCGCGGCAAAAAGCGGTC3′).

INTRACELLULAR CYTOKINE STAINING/FLOW CYTOMETRY

For *ex vivo* T cells assays, 7–9 days post MUC1 vaccination, mice were sacrificed and spleens were removed. Single-cell suspensions were made by mashing the spleens through a 40-μm filter. Total T cells were then bead isolated (Pan T Cell Isolation Kit II, Miltenyi) and cultured with day 6 MUC1-loaded BMDC (prepared as described in "MUC1 vaccination") for 4–6 h in the presence of GolgiStop (BD biosciences). Cells were then stained with the indicated antibodies using the BD Cytofix/Cytoperm™ kit (BD Bioscience) according to the manufacturer's protocol. All samples were run on a Fortessa (BD bioscience) flow cytometer and analyzed using FACSDiva (BD Bioscineces) and FlowJo software (Tree Star Inc.). Antibodies used: CD3-PerCP, CD11c-BV421, CD80-FITC, CD86-APC/Cy7, CD40-APC, CD3-PeCy5, CD4-V450, CD8-AF700, IFNγ-PeCy7, TNFα-PE, IL-2-APC, CD44-FITC, CD3-APC/Cy7, and CD8 PerCP.

PHOSPHOFLOW

Twenty-four hours following MUC1 vaccination, splenocytes were harvested as above. Post isolation, cells were put into AIM-V with or without 30 ng/mL IL-10 (PeproTech) for 20 min. At the end of culture, cells were immediately fixed in 1.6% PFA for 10 min at room temperature. After 10 min, four volumes of ice-cold methanol were added and samples were stored at −80°C. At the time of staining cells were put at room temp for 10 min and then immediately spun down and resuspended in flow buffer (PBS containing 1% BSA, 0.02% sodium azide, and 2 nM EDTA). After 10 min incubation at room temperature, cells were spun down and washed with flow buffer twice. Samples were then stained with antibodies against cell surface antigens CD11c, NK1.1, and CD3 and phospho-specific anti-pSAT3 antibody for 1 h at room temperature and prepared for analysis via standard protocol and as described above. Antibodies used: CD11c-Pacific Blue, pSTAT3-AF647, NK1.1-PE, and CD3-APC/Cy7.

EX VIVO DC STIMULATORY CAPACITY ANALYSIS

MUC1 transgenic mice were pretreated with antibodies and vaccinated as in "IL-10R Blockade." Post-vaccination, DC were bead isolated (CD11c MicroBeads, Miltenyi) from the spleens of the vaccinated animals. These DC were put into culture with bead isolated (CD4 T cell Isolation Kit II, Miltenyi) CFSE stained MUC1 specific VFT CD4 T cells (15) at a ratio of 1 DC to 5 VFT cells in complete DMEM. Twenty-four hours after the start of culture half of the media was removed and saved for cytokine analysis. IL-2 was analyzed by ELISA (BD OptEIA Mouse IL-2 ELISA set, BD) according to the manufacturer's protocol. The media was replaced with fresh cDMEM and the cultures were allowed to incubate for three more days. T cell proliferation was then analyzed by CFSE dilution.

ELISPOT

Millipore MultiScreen® Filter Plates (Millipore) were pretreated according to the manufacturer's instructions using the Mouse IFNg ELISPOT kit (Mabtech). Bead isolated CD4 and CD8 T cells (CD4 T cell Isolation Kit II and CD8α Isolation Kit II, Miltenyi) were cultured as above (see Intracellular Cytokine Staining/Flow Cytometry) with MUC1 pulsed BMDC and analyzed according to the established protocol. DC alone, media alone, and T cells alone were used to establish background cytokine production.

STATISTICAL ANALYSIS

Where appropriate, statistical significance was determined by performing an unpaired Student's *t*-test. *Denotes a p-value <0.05 and **denotes a p-value of <0.01. When indicated, to allow for pooling of data from multiple experiments, values have been transformed to account for minor variations in instrument settings and other potential sources of variation (i.e., minor batch to batch variance in DC vaccine prep, etc.). Briefly, all experimental values were

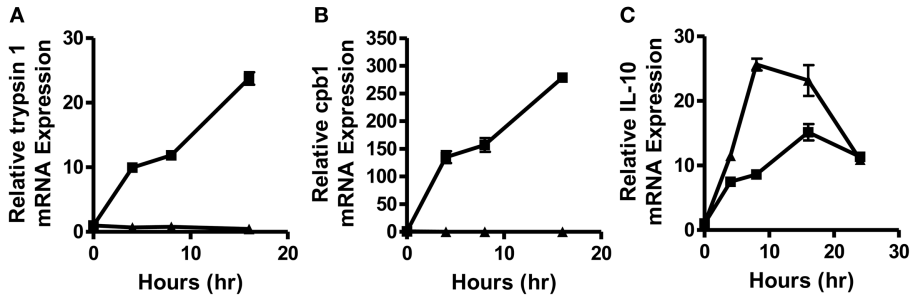

FIGURE 1 | Splenic DC activation is suppressed as early as 4–8 h post-vaccination with a self-, but not a foreign-antigen and correlated with early IL-10 production in the spleens of these animals. WT (squares) and MUC1.Tg mice (triangles) were vaccinated with MUC1p plus Poly-ICLC via tail vein. Spleens were removed at indicated hours post-vaccination and total splenic mRNA levels of trypsin 1 **(A)**, carboxypeptidase B1 (CPB1) **(B)**, and IL-10 **(C)** were determined relative to the control gene HPRT. Values shown represent expression relative to the baseline expression in mice of that genotype (WT and MUC1.Tg) at 0 h post-vaccination. Data are representative of three pooled mice were group per time point shown. Data points show mean ± SEM of three technical replicates and are representative of two independent experiments.

divided by the mean value of the control group from the experiment in which they were run. "Relative" values therefore represent a standardized deviance from control.

RESULTS

IL-10 EXPRESSION IN THE SPLEEN IS INCREASED 4–8H POST-VACCINATION WITH MUC1p AS SELF-ANTIGEN AND CORRELATES WITH DC SUPPRESSION

In order to determine how quickly post-vaccination with a self- versus a foreign-antigen DC phenotype and function begin to diverge and to obtain a more accurate picture of what factors might be responsible for supporting this divergence, we vaccinated intravenously WT and MUC1.Tg mice with the MUC1 100mer peptide (MUC1p) admixed with the Poly-ICLC adjuvant. MUC1.Tg mice express the human tumor antigen MUC1 under the control of its endogenous promoter and therefore MUC1p is seen as a self-antigen in these mice, whereas it is seen as a foreign-antigen in WT animals. Mice were sacrificed 4, 6, 8, and 16 h post-vaccination and the spleens removed for mRNA isolation and analysis. As early as 4 h post-vaccination, two newly discovered biomarkers of DC activation, trypsin 1 and CPB1 (12), were up-regulated in the spleens of WT mice but suppressed in MUC1.Tg mice (**Figures 1A,B**). In addition to differences in the levels of these enzymes, which our previous study showed to be expressed only in DC and representative of a larger cohort of "pancreatic" enzymes that robustly activated DC expression, we also detected at this early-time point higher levels of IL-10 mRNA in the spleens of vaccinated MUC1.Tg mice compared to WT mice. At 24 h post-vaccination and later, IL-10 production was at equal levels in self- and foreign-antigen vaccinated mice (**Figure 1C** and data not shown).

DC FROM SPLEENS OF MUC1p (SELF-ANTIGEN)-VACCINATED MUC1.Tg MICE ARE MORE SENSITIVE TO IL-10 THAN MUC1p (FOREIGN-ANTIGEN)-VACCINATED WT MICE

The above data showing differences in IL-10 levels early post-vaccination but no difference at 24 h and later would indicate a modest and transient effect by IL-10 on DC. This was, however, inconsistent with our previous observations that functional differences between DC post self-antigen versus foreign-antigen vaccine were evident as late as 72 h post-vaccination (12). We considered the possibility that the early action of IL-10 on DC, along with other factors, might increase their sensitivity to IL-10 at the later-time points. To query this, DC were removed from the spleens of WT and MUC1.Tg mice 24 h post MUC1p vaccination and exposed to IL-10. As signaling through the IL-10R is known to occur through a STAT3 intermediate, the sensitivity of DC to IL-10 was assessed by phosphoflow, measuring phospho-STAT3 levels post *ex vivo* exposure to IL-10. As hypothesized, there was a significant increase in the number of DC showing STAT3 phosphorylation as well as higher levels of pSTAT3 in the spleens of MUC1p-vaccinated MUC1.Tg mice (**Figures 2A–C**) indicating that DC in the spleens of MUC1.Tg mice are not only exposed to more IL-10 early on, but are also more sensitive to it at the later-time points.

IL-10R BLOCKADE INCREASES COSTIMULATORY MOLECULE EXPRESSION ON DC FOLLOWING VACCINATION WITH MUC1p AS SELF-ANTIGEN

Given the inverse correlation between IL-10 production and DC pancreatic enzyme expression in the first 24 h following vaccination and previously published data showing that IL-10 is necessary for suppression of trypsin 1 and CPB1 following vaccination with a self-antigen (12), we hypothesized that blocking IL-10 signaling in self-antigen vaccinated mice would improve DC activation and costimulatory molecule expression. We injected MUC1.Tg mice with an antibody against IL-10R and vaccinated intravenously 24–48 h later with MUC1p plus Poly-ICLC. At 24 h post-vaccination, the surface phenotype of splenic DC was analyzed by flow cytometry. As hypothesized, there was an increase in the level of cell surface expression of CD40, CD80, and CD86 in DC from mice pretreated with the antibody to IL-10R, but not from mice treated with the isotype control antibody (**Figures 3A–C**). Increases in CD40 and CD86 were statistically significant, which is of interest because these two molecules were shown previously to be specifically inhibited in mice vaccinated with a self- but not a foreign-antigen (12). In addition to being less active as measured by surface marker expression, these DC are also less capable of stimulating MUC1 specific CD4 T cells *in vitro*. DC isolated from MUC1.Tg mice

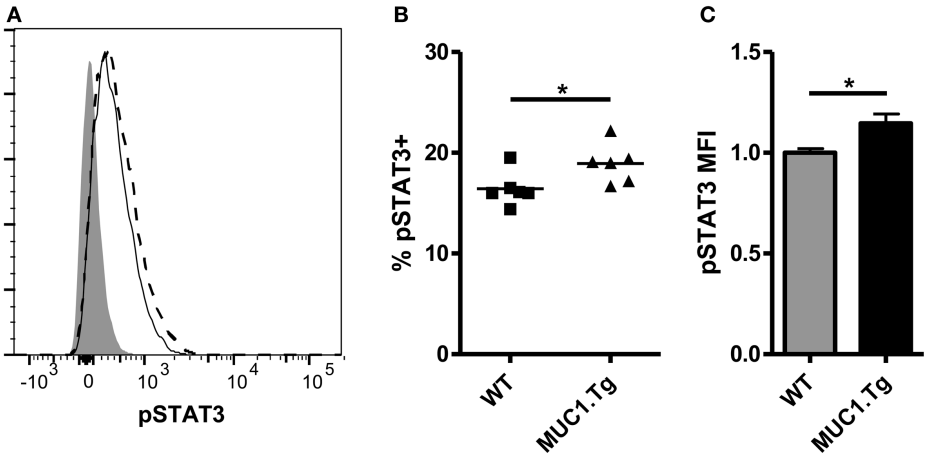

FIGURE 2 | Dendritic cell from spleens of mice vaccinated with self-antigen have higher levels of phosphorylated STAT3 after IL-10 treatment than DC from spleens of mice vaccinated with foreign-antigen. WT (solid line) and MUC1.Tg (dashed line) mice were vaccinated with MUC1p via the tail vein. Twenty-four hours post-vaccination splenocytes were removed and treated with 30 ng/mL of IL-10 for 20 min. Following incubation, cells were fixed and phospho-STAT3 expression in CD11c+NK1.1− splenocytes was analyzed via phoshpoflow. **(A)** A representative flow plot is shown. The shaded histogram represents the fluorescence level when cells are treated with standard surface markers and an isotype-matched control instead of the phosphospecific antibody. pSTAT3 positivity **(B)** and MFI **(C)** were analyzed. In **(B)** symbols correspond to individual animals and are representative of two independent experiments. **(C)** Values shown have been normalized to the expression level of the control group (WT) in order to allow for pooling of data from separate experiments run on multiple days. Bars are representative of nine mice from two combined experiments and show the mean ± SEM. *Indicates a *p*-value of <0.05.

pretreated with an antibody against the IL-10R prior to MUC1 vaccination and put into culture with MUC1 specific CD4 T cells induce higher levels of IL-2 (**Figure 4A**) and CD4 T cell proliferation (**Figures 4B,C**) compared to DC from MUC1.Tg mice pretreated with an isotype-matched control antibody.

BLOCKING IL-10 SIGNALING PRIOR TO VACCINATION WITH MUC1p AS SELF-ANTIGEN IMPROVES CD4 T CELL RESPONSE

The increase of costimulatory molecule expression when IL-10 signaling was blocked just prior to vaccination suggested that there would be a resultant increase in the T cell response. To test this, we again pretreated mice with an anti-IL-10R antibody or an isotype-matched control and injected with a vaccine composed of DC loaded with MUC1p. We chose the DC-based vaccine expecting that it would optimally stimulate both CD4 and CD8 T cells, as has been previously shown (16). Seven to nine days post-vaccination, splenic T cells were isolated and their production of relevant cytokines analyzed by ELISPOT and intracellular flow cytometry. In MUC1.Tg mice treated with anti-IL-10R, there was a significant increase in MUC1p specific, IFNγ+ CD4 T cells when compared to mice treated with an isotype-matched control antibody (**Figures 5A,C**). The level of the response was equivalent to the response of WT mice pretreated with the isotype control antibody (**Figure 5A**). There was no increase over the isotype control of the T cell response in WT mice pretreated with the anti-IL-10R antibody (**Figures 5A,C**), indicating that the effect of IL-10 we saw in MUC1.Tg mice was specific for controlling responses to self- but not foreign-antigens. There was a small but not significant increase in the CD8 response that was detectable only by the more sensitive ELISPOT (**Figures 5B,D**).

DISCUSSION

Vaccines against cancer have garnered a lot of attention in recent years. Much of this was sparked by the relatively recent approval of Sipuleucel-T, the first vaccine to show survival benefit in a solid metastatic tumor (17, 18). Implementation of Gardasil®, a quadrivalent human papilloma virus specific vaccination intended to prevent cervical cancer in women (19, 20) has also sparked new efforts in designing prophylactic cancer vaccines not just for viral cancers but for many tumor types (21–24). Most non-viral tumor antigens fall into the category of self- or altered self-antigens. Mounting an effective immune response against them represents a unique challenge. One must design vaccines that overcome the natural tolerizing forces acting on responses to self-antigens, while minimizing adverse autoimmune effects.

Our work with the MUC1 tumor antigen in the MUC1.Tg mouse model system has shown that hyporesponsiveness to the MUC1 peptide vaccines in these mice is neither due to the elimination of MUC1 peptide-specific T cells by central tolerance, nor solely to their conditioning in the periphery, but rather by the control of their activation (15). Indeed, even when unconditioned MUC1 specific T cells are transferred into MUC1.Tg hosts, they are hyporesponsive to MUC1 peptide vaccination but respond vigorously in WT hosts. Most recently, we determined that the major reason for the lack of T cell response is profound, albeit transient, tolerization of DC in MUC1p-vaccinated MUC1.Tg mice early post-vaccination (12). Here, we show that this is likely due to the very early and exaggerated effect of IL-10 on these DC in the first 4–24 h post-vaccination. IL-10 is known to reduce MHC Class II and costimulatory molecule expression on DC (4–6), DC motility (25, 26), and overall T cell stimulatory capacity (27, 28), all

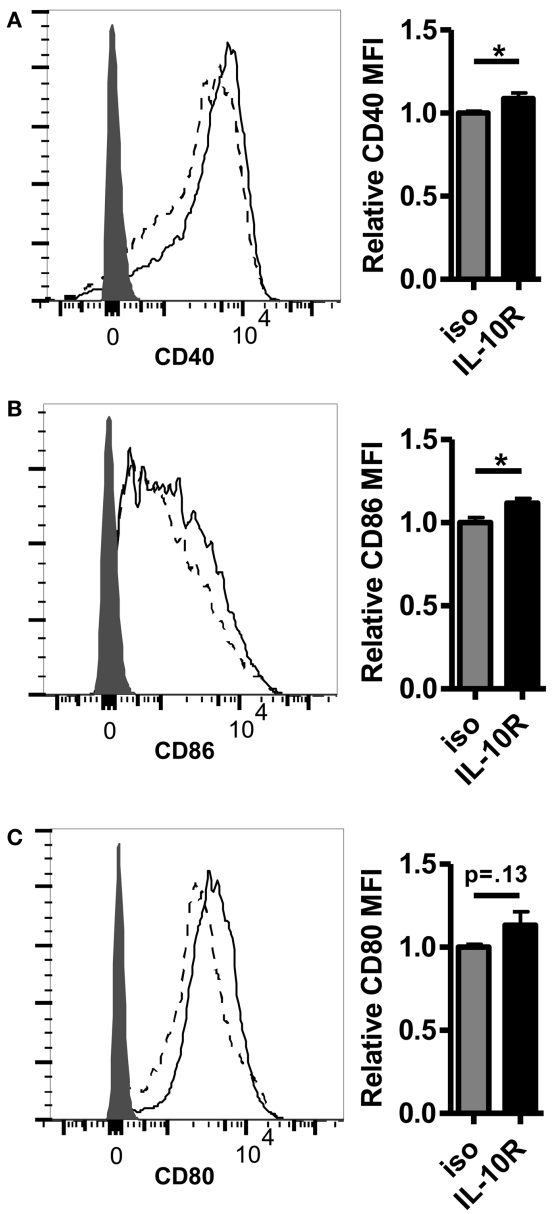

FIGURE 3 | Pretreatment with an antibody against the IL-10 receptor increases the level of costimulatory molecule expression on DC in the spleens of self-antigen vaccinated mice. MUC1.Tg mice were pretreated with an antibody against the IL-10 receptor (IL-10R, solid lines) or were given a non-specific isotype control (iso, dashed lines). One to two days later they were vaccinated as in **Figure 1** and 24 h post-vaccination, splenocytes were removed and analyzed via flow cytometry. The expression level of CD40 **(A)**, CD86 **(B)**, and CD80 **(C)** on splenic DC (CD11C+, MHC Class II+) was determined. Shaded histograms represent fluorescence in samples stained with isotype alone. Bar graph values shown have been normalized to the expression level of the control group (iso) in order to allow for pooling of data from separate experiments run on multiple days. **(A,C)** Data are combined from two independent experiments and representative of six mice. **(B)** Data are combined from three independent experiments and are representative of 10 mice. Bars represent mean ± SEM. *p*-Values are as stated unless designated by a *, which indicates a *p*-value of <0.05.

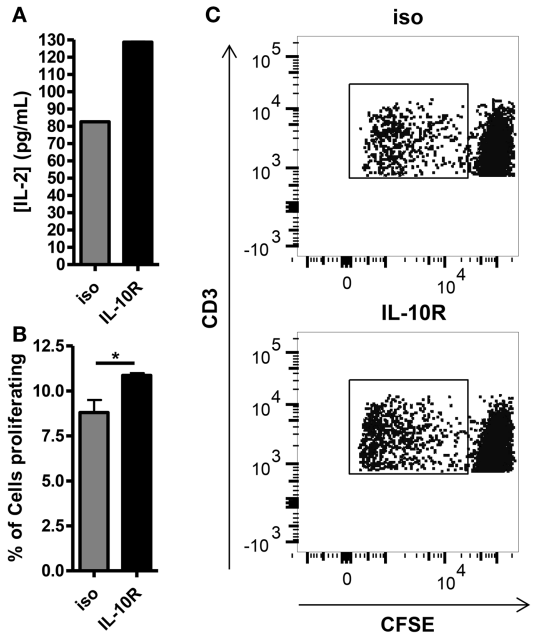

FIGURE 4 | Blocking of the IL-10 receptor prior to intravenous MUC1 peptide vaccination increases the ability of splenic DC from MUC1.Tg mice to stimulate MUC1 specific CD4 T cells *ex vivo*. MUC1.tg mice were treated as in **Figure 3**. Twenty-four hours post MUC1 vaccination, splenocytes from three to four mice per treatment group were pooled and bead isolated DC from these pooled splenocytes were put into culture with CFSE labeled MUC1 specific CD4 T cells (VFT cells) at a ratio 1DC:5VFT. Twenty-four hours after the start of culture, half of the culture media was removed and the concentration of IL-2 was measured by ELISA **(A)**. Cultures were allowed to incubate three more days for a total of four and VFT proliferation was analyzed by CFSE dilution **(B,C)**. **(B)** Bars represent the mean percentage of CD3+CD4+ T cells that had proliferated at 4 days of three technical replicates ±SEM. **(C)** A representative flow plot is shown. Data are representative of two to three independent experiments. *Indicates a *p*-value of <0.05.

of which are characteristics of DC in the spleens of MUC1p-vaccinated MUC1.Tg mice (12).

The effects of IL-10 on vaccines have been observed previously. In the therapeutic setting, IL-10R blockade alone or along with vaccination can improve Th1 responses and enhance pathogen clearance (29–31). In a prophylactic setting, mice given the BCG vaccination for prevention of *Mycobacterium tuberculosis* show improved Th1 responses and enhanced resistance to pathogen challenge when IL-10R is blocked at the time of vaccination (32). In this paper, we describe a distinct new role for IL-10 in impacting vaccine outcome that is unique in its specificity for self-antigen. The same MUC1 peptide, given as a self-antigen to MUC1.Tg mice but as a foreign-antigen to WT mice, causes only low levels IL-10 production in WT mice and increased production in MUC1.Tg mice. In previously published studies showing improvement in immune responses after IL-10R blockade, IL-10 was produced in response to acute or persistent pathogen infections, whereas in this case it was specifically triggered in response to the presence of a self-antigen or specifically inhibited in the presence of a foreign-antigen.

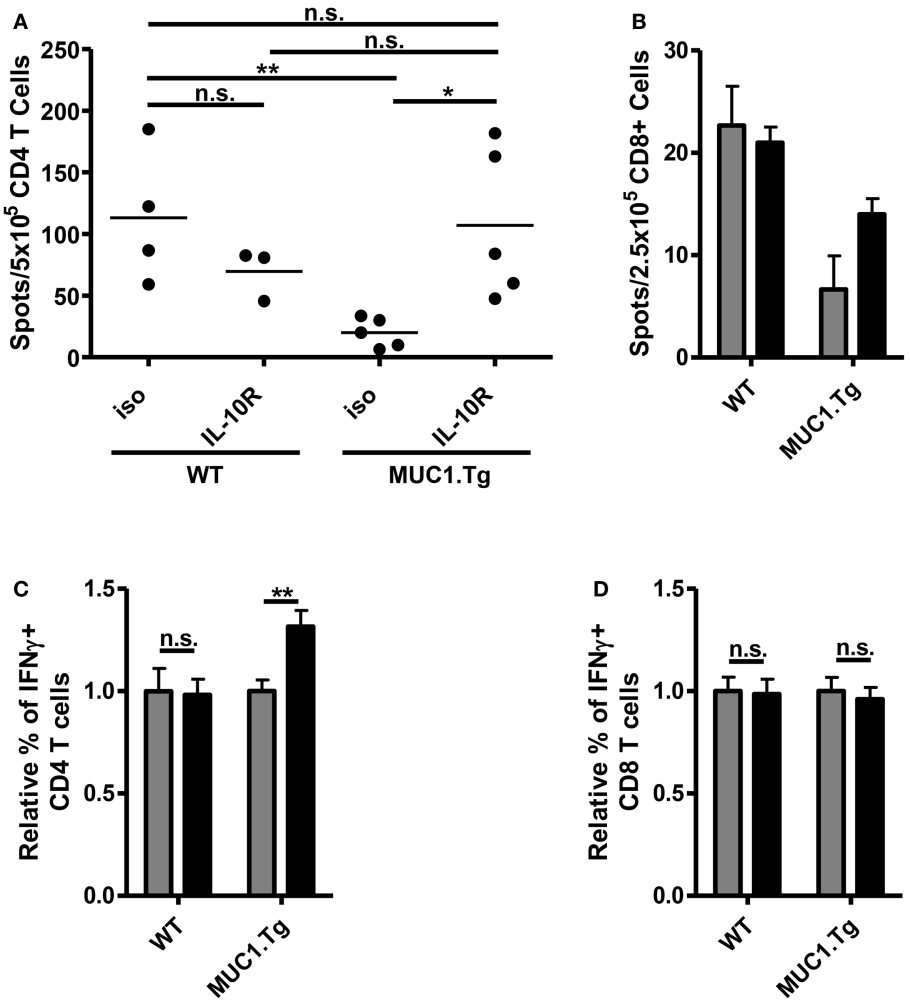

FIGURE 5 | Treatment with anti-IL-10R antibody at the time of vaccination increases the number of MUC1p specific, IFNγ+ CD4 T cells without an effect on CD8 T cells. WT and MUC1.Tg mice were pretreated with an antibody against the IL-10 receptor (IL-10R, black bars) or a non-specific isotype control (iso, gray bars). One to two days following antibody treatment, mice were vaccinated with DC loaded with MUC1p. Seven to nine days post-vaccination, spleens were removed and bead isolated CD4 **(A,C)** and CD8 T cells **(B,D)** were cultured with MUC1p loaded bone marrow derived DC overnight and analyzed by ELISPOT **(A,B)**, or were cultured for 6–8 h in the presence of brefeldin-A and analyzed by intracellular flow cytometry **(C,D)**. **(A)** Data are combined from two independent experiments with each spot indicating an individual animal. Data are representative of three independent experiments. **(B)** Bars indicate the average of three technical replicates pooled from three individual animals per group. Data are representative of two independent experiments. **(C,D)** Values shown are normalized to the response of mice of that genotype (WT versus MUC1.Tg) given the control treatment (iso). Data are combined from two independent experiments and are representative of five to six mice per group. Bars represent mean ± SEM. *Indicates a p-value of <0.05; **indicates a p-value of <0.005.

We have yet to identify the source in the spleen of this early IL-10 production in self-antigen vaccinated mice. Every cell of the immune system can produce IL-10 given proper stimulation. However the kinetics and pattern of IL-10 production in MUC1p-vaccinated MUC1.Tg mice limits the possibilities considerably. The fact that IL-10 production was antigen dependent suggests a cell of the adaptive immune system. Regulatory T cells have previously been shown to be important in preventing MUC1p specific immune responses in MUC1.Tg mice (12, 33). However, preliminary experiments have been unable to identify IL-10 producing regulatory T cells in MUC1.Tg mice at rest or immediately following vaccination (data not shown), as has been shown in some models after self-peptide administration (34). Given that regulatory T cells can modulate the function of a wide variety of innate cells, including NK cells (35, 36) and DCs (37, 38), it is possible that through secretion of another cytokine or through direct interactions, they induce IL-10 production either directly or indirectly in another cell population.

Irrespective of the source, the self-antigen specific role of IL-10 reported in this paper supports IL-10 inhibition as a way of improving the efficacy of vaccines against self-antigens that are candidate tumor antigens. While our major success in this study was in improving CD4 T cell responses, we would hypothesize that CD8 T cell responses generated upon boosting would

be improved as well in these animals as a consequence of generation of a larger population of helper CD4 T cells that are required for effective CD8 T cell memory differentiation (39, 40). The concern remains that any manipulation leading to enhanced responses to self/tumor antigens might cause adverse autoimmune reactions. However, current research has shown this concern can be addressed by proper antigen selection. For example, vaccines against self/tumor antigens MUC1 and α-lactalbumin have shown clinical and preclinical efficacy with no induction of autoimmunity (23, 41, 42). And vitiligo, caused by successful anti-melanoma vaccines is an autoimmune event that can be easily tolerated (43–45). Furthermore, while long term IL-10 deficiency can cause adverse autoimmune effects (46, 47), our data suggest that in order to improve the vaccine response, IL-10 would need to be blocked only transiently at the time of initial vaccination.

AUTHOR CONTRIBUTIONS

The research reported in this article was conducted by Douglas M. Marvel. Douglas M. Marvel and Olivera J. Finn jointly designed the experiments and prepared the manuscript.

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Stressful presentations: mild cold stress in laboratory mice influences phenotype of dendritic cells in naïve and tumor-bearing mice

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The ability of dendritic cells (DCs) to stimulate and regulate T cells is critical to effective anti-tumor immunity. Therefore, it is important to fully recognize any inherent factors which may influence DC function under experimental conditions, especially in laboratory mice since they are used so heavily to model immune responses. The goals of this report are to 1) briefly summarize previous work revealing how DCs respond to various forms of physiological stress and 2) to present new data highlighting the potential for chronic mild cold stress inherent to mice housed at the required standard ambient temperatures to influence baseline DCs properties in naïve and tumor-bearing mice. As recent data from our group shows that $CD8^+$ T cell function is significantly altered by chronic mild cold stress and since DC function is crucial for $CD8^+$ T cell activation, we wondered whether housing temperature may also be influencing DC function. Here we report that there are several significant phenotypical and functional differences among DC subsets in naïve and tumor-bearing mice housed at either standard housing temperature or at a thermoneutral ambient temperature, which significantly reduces the extent of cold stress. The new data presented here strongly suggests that, by itself, the housing temperature of mice can affect fundamental properties and functions of DCs. Therefore differences in basal levels of stress due to housing should be taken into consideration when interpreting experiments designed to evaluate the impact of additional variables, including other stressors on DC function.

Keywords: cold stress, thermoregulation, norepinephrine, mouse models of cancer, anti-tumor immunity

INTRODUCTION

Dendritic cells (DCs) play a vital role in the generation of effective and long-term immune protection from cancer and other diseases. DCs are antigen presenting cells, which educate tumor-specific T cells and provide signals for T cell proliferation and expansion (1, 2). Importantly, DCs bridge the innate and adaptive immune responses so their presence and functional capacity affect both arms of anti-tumor immunity (3, 4). Properties of DCs that are investigated to determine their stage of development include surface expression of major histocompatibility complex (MHC) class II molecules and co-stimulatory CD86 as well as cytokine production. Additionally, DCs are also being used clinically in cancer vaccines (5, 6) and this approach has rendered promising results; however, considerable room for improvement remains (7–9).

In addition to anti-tumor immunity and immune surveillance, DCs also participate in tolerizing the immune system to tumor antigens, which can render the anti-tumor immune response ineffective (10). Cross-presentation, a process that DCs undergo in order to activate $CD8^+$ T cells, plays a major role in generating anti-tumor immunity (11), however; when DCs of tumor-bearing hosts undergo this vital process, T cell tolerance often results (5). Recently, it has been reported that DCs able to up-regulate MHC II (signal 1) in the absence of CD86 (signal 2) become tolerogenic DCs (12, 13). Although considerable progress has been made toward understanding how DCs become tolerogenic (10, 14, 15), the precise mechanisms by which tumors modulate cross-priming to suppress the $CD8^+$ T cell response remain largely unknown. This incomplete understanding of the role DCs play in immune evasion remains a vital question as DCs are being actively investigated in mouse models to help reveal their role in the anti-tumor immune response. Therefore, it is important to fully recognize the impact of any inherent physiological factors in mice, which can alter DC function and to understand the impact these factors could have on experimental models of antigen presentation and immunotherapy.

We have been interested in the effects of various types of biologically relevant stress on the functional properties of immune cells (16) and have previously reported on the impact of mild (fever-range) heat stress on DC function (17, 18). It is important to note that there are a wide variety of stressors including physical, environmental, and emotional forms of stress that can alter homeostasis in cells or in the whole organism (19). Two major hormonally driven mechanisms are believed to mediate the influence of stress on the immune response. Glucocorticoids are released following stress leading to increased glucose metabolism necessary to provide extra energy to combat that stressor. Additionally, catecholamines, such as norepinephrine (NE), are released from sympathetic nerves and bind receptors on immune

cells thereby impacting the immune response. Both of these hormonal mediators can influence immune processes including cell proliferation, migration, and cytokine production (20). Here, we first briefly summarize some of the previous work done to investigate the more specific effects of stress on DC function. While some studies show that acute, short term, stress may enhance DC function *in vitro*, resulting in a better ability to prime naïve T cells, other studies, particularly those which utilize the addition of exogenous stress hormones, reveal that stress impedes DC function *in vivo*. We outline reports suggesting a vital role of the stress hormone NE on DC function *in vivo* but not *in vitro*. We also summarize literature showing beneficial effects of a mild thermal stress on DC function both *in vitro* and *in vivo*. Finally, we report that when mice used to investigate DC function are housed at standard ambient temperatures they experience an underappreciated form of chronic physiological cold stress that alters the baseline used to understand the impact of experimental stressors or other treatments on DC function. We suggest that chronic mild cold stress, similar to other forms of stress inherent to mouse caging conditions including stress caused by lack of exercise and overeating (21), should be taken into consideration when assessing baseline properties of DCs in naïve or tumor-bearing mice.

STRESS CAN TARGET DC FUNCTION

Dendritic cells have already been the subject of many studies investigating the impact of stress on immune function. Acute stressors, lasting minutes to hours, have been shown to augment DC function as seen by enhanced maturation and increased trafficking from skin to lymph nodes (22, 23). Prior to immunization, specific kinds of acute stress, such as psychological stress induced by placing mice in restraints or on a slow moving shaker works as an adjuvant leading to increased DC migration from the skin to the lymph nodes and also improves antigen-specific T cell priming (24, 25). The impact of such acute psychological stress on DCs has also been investigated in humans. Social stress in human participants (induced by public speaking) generates a decrease in skin DCs, which the authors suggest indicates that these cells have trafficked to the lymph node (26) where they are available to interact with T cells and initiate immune activation. However, while some stressors may elicit beneficial effects on DC function and general immunity, chronic or excessive exposure to stress is generally thought to negatively influence immune function (27). Many studies, particularly those using exogenous administration of glucocorticoids, stress hormones which signal to turn down immune activity, suggest inhibitory effects of stress on DC function (28, 29). Both oral (30) and topical (31) application of glucocorticoids leads to a marked reduction in DC numbers. Many studies specifically investigate the impact of dexamethasone (DEX), a commonly prescribed glucocorticoid, on DC development and function. It has been shown that DEX greatly reduces epidermal DC numbers in mice (32, 33) as well as in the spleen, lymph node, and liver (34, 35). DEX treatment also limits DC migration to the draining lymph node (36). Additionally, DEX is correlated with reduced expression of surface maturation markers on DCs including CD86 and MHC class II (35, 37, 38). *In vitro*, DEX treatment reduces the ability of bone marrow (39, 40) and skin derived DCs (32, 41) as well as a murine epidermal DC line (42) to stimulate T cells. DEX also impairs antigen presentation by DCs reducing T cell activation *in vivo* (35). It has also been shown that following DEX treatment, DCs are unable to fully mature and these immature DCs induced a subpopulation of immunosuppressive regulatory T (T_{reg}) cells (39). Additionally a reduction of interleukin (IL)-1β and IL-12p70 secretion from DCs has been shown following DEX treatment (37). Compared to control cells, glucocorticoid-treated DCs produce less granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-alpha (TNF-α) and IL-1α, all cytokines required for survival and maturation, and are less apt to initiate antigen presentation and migration (43). Further, treatment with other glucocorticoids (hydrocortisone or clobetasol) led to DC apoptosis identified by DNA damage, caspase-3 activity, and CD95 up-regulation (43).

Catecholamines, such as NE and epinephrine, also play an important role in mediating the relationship between stress and DCs. Manipulating the function of stress induced catecholamines has been linked to altered DC function (44). For example, when healthy patients were administered a β-adrenergic agonist (oral salbutamol), which mimics NE signaling, IL-12 production by DCs was decreased, inhibiting Th1 development (45). Another study found that NE similarly suppressed IL-12 production in a dose-dependent manner and that this was reversible with a glucocorticoid agonist, RU 486 (46). As Th1 and Th2 responses are mutually inhibitory, this leads to an increasingly prominent Th2 environment, which is defined by various immunosuppressive properties including inhibition of macrophage activation, T cell proliferation, and pro-inflammatory cytokine production (47). The effects of catecholamines may be most important to DCs in the early stages of antigen processing (44). Short term exposure of bone marrow-derived DCs to NE or epinephrine at the early stage of stimulation inhibits IL-12 and favors IL-10 production as well as a reduced ability to stimulate T cells (48). Skin DCs are also sensitive to catecholamine signaling. *In vitro*, treatment with NE, epinephrine, or β-adrenergic agonist (isoproterenol) hindered skin DCs from presenting antigen and this effect was reversed by treatment with ICI 118,551, a β_2-adrenergic antagonist (49). DC migration is NE dependent as demonstrated by decreased DC migration *in vivo* following NE depletion with 6-hydroxydopamine treatment (25). Additionally, NE has been shown to enhance phosphatidylinositol 3-kinase mediated antigen uptake by DCs (50). Taken together, these reports suggest that although some types of stress may benefit DCs under certain circumstances, it is generally accepted that chronic stress dampens many aspects of DC function.

EFFECTS OF MILD HYPERTHERMIA ON DCs

Environmental conditions have long been manipulated to create physiologically relevant stress. Thermal stress, induced when environmental conditions are either too hot or too cold to allow basal metabolism to maintain normal body temperature, is a classically studied stress in mice and humans (51). While conditions of severe heat or cold can be quite damaging to immunity, mild heat stress has been studied for its positive effects since ancient times because of its potential relationship to fever (52–55). In response to infection, body temperature increases to varying extents among different animals, but in all cases, homeostatic functions shift toward producing and conserving heat (52).

Generally, temperature elevation during fever ranges between 1 and 5°C above normal body temperatures (56–58). The physiological effects of fever have been mimicked experimentally by using mild hyperthermia treatments in mice, where body temperature is temporarily raised to fever-range (16). Many studies, including those from our own group, have examined how mild hyperthermia affects DCs and their function (17, 59–64).

Dendritic cell maturation is determined by the up-regulation of surface markers including MHC class II and CD86 (65, 66). Mild thermal stress increases levels of both of these markers on DCs (59). *In vitro* heating accelerates DC maturation as demonstrated by up-regulation of both CD86 and MHC class II (60, 61, 67). *In vivo* studies have also shown that up-regulation of both MHC class II and CD86 molecules on the surface of DCs from mice treated with whole body hyperthermia (61, 68). Additionally, hyperthermia in combination with other treatments including ionizing radiation (69), magnetic nanoparticles (70, 71), radiofrequency ablation (72) and vaccination (73, 74) results in enhanced DC function.

Dendritic cell migration to the lymph node is an important function required for efficient antigen presentation and our group and others have shown that mild heat stress can promote migratory activity of DCs. DCs in ear skin subjected to thermal stress in culture show increased migration compared to control samples (75), while increased DC migration into the lymph nodes of thermally stressed mice has also been demonstrated (61).

Heat treatment results in improved stimulatory function of DCs (59, 63, 67). Heated OVA-loaded DCs induce greater interferon-gamma (IFN-γ) responses from SINFEKL-specific T cells (64). Heat-treated SINFEKL pulsed DCs elicit greater antigen-specific $CD8^+$ T cell proliferation than unheated DCs (67). Heat also enhances the ability of DCs to cross-present to $CD8^+$ T (76) and activates $CD4^+$ T cells leading to antigen dependent memory (77). Additionally, mild hyperthermia alters the production of cytokines and chemokines from DCs, which are important for ensuring effective T cell priming. Mild heating increases DC production of inflammatory cytokines including IFN-γ, IL-17, IL-10, IL-12, and TNF-α (60, 61, 63). Taken together, the growing body of literature describing the effects of mild heat stress on DCs indicates that mild heat stress enhances DC function by promoting maturation and migration and increasing inflammatory cytokine production to assist with mediation of T cell priming to elicit T cell proliferation.

EFFECTS OF COLD STRESS ON DCs

We have summarized some of the previously reported complex effects of stress on DCs, including the general beneficial effects of temporary mild hyperthermia. We wondered whether the baseline function of DCs in these types of studies is influenced by ambient temperature used to house mice in research facilities. Laboratory mice are under a mild, yet constant cold stress as they are group housed at a cool (sub-thermoneutral) temperature (78–80). Additionally, since laboratory mice are provided with unlimited access to food and housed in small cages, which do not allow adequate room to exercise, they also experience additional metabolic stresses (21). Although these stressors have been identified as being important in other fields of research, such as obesity (81), they are not generally accounted for in the field of cancer immunology.

The fact that mice are mildly, yet chronically, cold stressed is not determined simply by body temperature measurements. In fact, while body temperature appears normal (~37°C) for mice housed at standard ambient temperatures required for research facilities (55), thermal preference studies over many decades have shown that mice prefer a warmer housing temperature near thermoneutrality (57, 78, 82, 83) indicating the degree of cold stress prompted by such housing. The degree to which underlying chronic cold stress has impacted the interpretation of the effects of other types of stress on immune function remains to be determined. Importantly, NE is released in response to stressors, including cold stress and, as detailed above, has a very significant influence on DC function.

Recent literature has detailed the impact of chronic cold stress in mice. The relationship between cold stress and metabolism has been investigated and alterations in insulin production (84), NE secretion (85), function of uncoupling proteins (81), and energy expenditure (86, 87) have been identified. Developmental and behavioral effects including differences in limb and tail length (88), cardiac tone and heart rate (89), and sleep (90) have also been observed when comparing cold stressed to non-stressed mice. Most recently, our group has shown that mild cold stress associated with standard housing conditions negatively impacts $CD8^+$ T cell dependent anti-tumor immune responses (55). To test whether DC function is influenced by chronic cold stress, we studied the impact of sub-thermoneutral housing temperatures on DC phenotype and function comparing the results to that seen from mice housed at thermoneutrality. Importantly, core body temperature in both groups of mice is the same, as shown previously (55).

We examined splenocytes from tumor-free and 4T1 tumor-bearing mice housed at standard (ST; 22°C) and thermoneutral (TT; 30°C) temperature. Because at 30°C the metabolic cold stress is greatly reduced, these mice represent un-cold stressed animals whereas their counterparts at 22°C are under chronic cold stress. We found that the number of splenocytes is similar in naïve (tumor-free) mice at ST and TT (**Figure 1A**). However, inoculation of mice with tumors induces an increase in splenocyte number at both ambient temperatures, however, this increase is larger at ST than at TT (**Figure 1A**). Confirming previous data (55), tumors grew slower in mice at TT compared to ST; tumor weight (**Figure 1B**) and volume (**Figure 1C**) were reduced in TT mice compared to ST mice. We also examined body weight for mice housed at each ambient temperature and found that prior to tumor inoculation mice at ST gained weight faster than mice at TT (**Figure 1D**). As tumors began to grow, mice at ST continued to gain even more weight than mice at TT (**Figure 1D**). These data show that animals housed at TT are physically smaller than those mice used as standard control models, while 4T1 tumor growth is accelerated in ST control mice.

We previously reported that spleens from mice at TT have fewer $CD11b^+GR\text{-}1^+$ myeloid derived suppressor cells (MDSCs) (55), so we wondered whether pan myeloid cells ($CD11b^+$) were similarly impacted by temperature (**Figure 2A**). We first determined that tumor-bearing mice at ST have significantly more $CD11b^+$ myeloid cells as well as a higher percentage of $CD11b^+$ cells

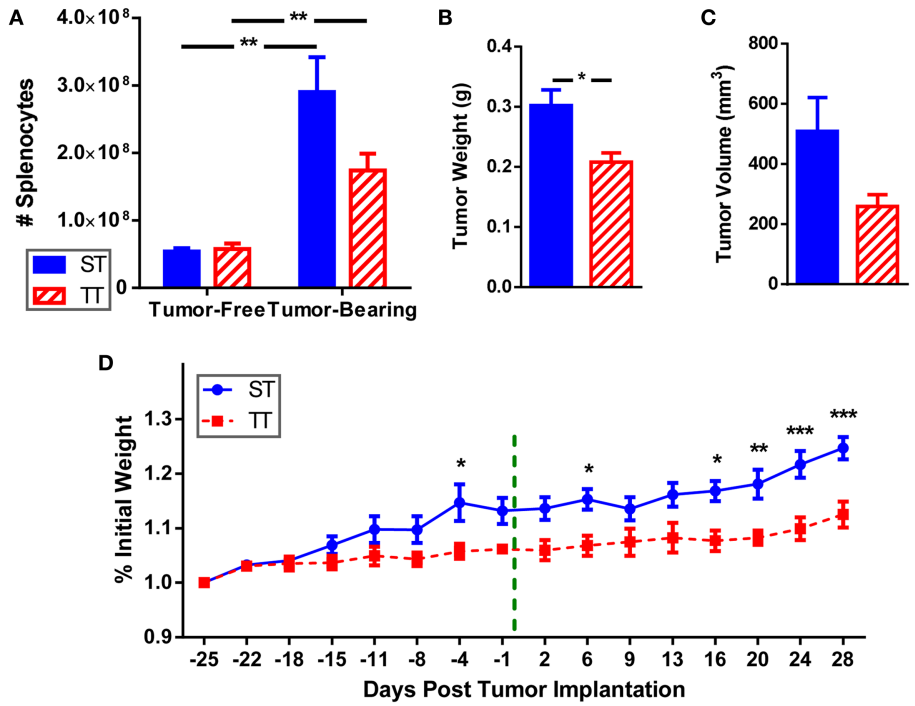

FIGURE 1 | Splenocytes, tumor size, and body weight are increased when mice are maintained at ST compared to TT. 4T1 tumor-bearing BALB/c mice and age-matched controls were maintained at ST or TT. **(A)** Splenocytes obtained from control and tumor-bearing mice were counted and **(B)** tumor weight and **(C)** volume were measured. Data presented as mean ± SEM; $n = 5$/group; Student's *t*-test; $^{*}p < 0.05$, $^{**}p < 0.01$. **(D)** Change in weight from the start of the experiment was measured. - - - - indicates day of tumor inoculation. Data presented as mean ± SEM; $n = 5$/group; two-way ANOVA with Bonferroni post-tests; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

compared to tumor-bearing mice at TT (**Figure 2B**). The number and proportion of splenic myeloid cells in tumor-free animals was unchanged (**Figure 2C**). These results suggest that the effects of 4T1 tumor growth on the accumulation of myeloid cells in the spleen may be overestimated in mice housed under standard conditions since the cellular increase is also dependent on ambient temperature.

It has been reported that DC numbers in cancer patients are reduced compared to healthy controls (91); thus, we next investigated numbers of splenic DCs in tumor-free and tumor-bearing mice maintained at ST and TT based on CD11c expression. Total DCs were identified as $CD11c^{+}$ cells. We found that absolute numbers of splenic DCs (**Figure 3A**) increased following tumor implantation in mice at ST but not at TT (**Figure 3B**). However, the proportion of DCs decreased at both ST and TT following tumor inoculation (**Figure 3C**). We next examined plasmacytoid DCs ($B220^{+}CD11c^{+}$) (**Figure 3D**) which, following stimulation, are major interferon producers (92). We discovered that absolute numbers of plasmacytoid DCs increase following tumor inoculation in mice at ST but not at TT (**Figure 3E**), whereas percentages significantly decrease following tumor inoculation in mice at TT but not at ST (**Figure 3F**). When we investigated non-plasmacytoid DCs ($B220^{-}CD11c^{+}$) (**Figure 3D**) (93–95), we again found that absolute numbers increase following tumor inoculation in mice at ST but not at TT (**Figure 3G**) but that percentages of these cells significantly decrease following tumor inoculation in mice at TT only (**Figure 3H**). These data demonstrate that the number of DCs found in the spleens of laboratory mice do not show the expected increase in numbers after tumor inoculation when mice are maintained at thermoneutrality. Thus, ambient temperature should be considered when interpreting data regarding immune cell subsets in the spleens from mice used for cancer immunology studies.

We further dissected the non-plasmacytoid cell population by quantifying a subset of immature ($MHCII^{-}CD86^{-}$) and two subsets of mature ($CD11c^{+}MHCII^{+}CD86$, $CD11c^{+}MHCII^{+}CD86^{-}$) cells among $CD8\alpha^{+}$ and $CD4^{+}$ non-plasmacytoid DCs (**Figure 4A**). $CD8\alpha^{+}$ DCs are major producers of IL-12, able to initiate a robust inflammatory response as well as efficiently presenting antigen to $CD8^{+}$ T cells (96–99). We found that both absolute numbers (**Figure 4B**) and percentages (**Figure 4C**) of immature $CD8\alpha^{+}$ non-plasmacytoid DCs are increased to a greater extent following tumor inoculation in mice at ST compared to TT. Absolute numbers of $CD86^{-}$ mature $CD8\alpha^{+}$ non-plasmacytoid DCs increased following tumor inoculation in mice at ST but not TT (**Figure 4D**). The percentage of $CD86^{-}$ mature $CD8\alpha^{+}$ non-plasmacytoid DCs decreased at ST but not TT following tumor inoculation (**Figure 4E**). $CD86^{+}$ mature $CD8\alpha^{+}$ non-plasmacytoid DCs were unchanged in absolute number (**Figure 4F**) but their proportion in the spleen was modestly, yet significantly decreased at both ST and TT following tumor inoculation (**Figure 4G**). The increased numbers of immature and $CD86^{-}$ mature $CD8\alpha$ non-plasmacytoid DCs present in mice at

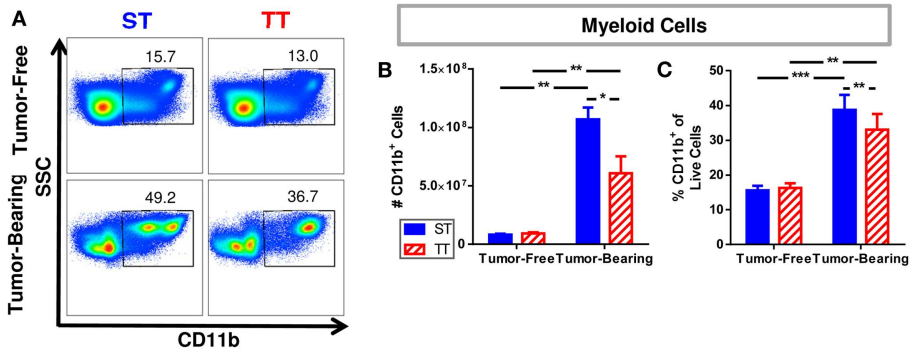

FIGURE 2 | Splenic myeloid cells are increased in tumor-bearing mice maintained at ST compared to TT. Single cell suspensions of splenocytes from 4T1 tumor-bearing mice and age-matched controls were stained for CD11b and analyzed by flow cytometry. **(A)** Representative dot plots from each group show the gating strategy used to select CD11b$^+$ cells. Percentage of cells are shown above their respective gate. **(B)** The absolute number of CD11b$^+$ cells calculated from the total number of splenocytes counted in each individual mouse. **(C)** The percentage of CD11b$^+$ cells of the total population of live cells as determined by DAPI staining. Data presented as mean ± SEM; $n = 5$/group; Student's *t*-test; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ST suggests that many of the DCs from these mice may not be able to become activated.

Further, we investigated the same subsets of immature and mature CD4$^+$ non-plasmacytoid DCs (**Figure 4A**). We also found a major increase in absolute numbers (**Figure 4H**) and percentage (**Figure 4I**) of immature CD4$^+$ non-plasmacytoid DCs in mice at ST but not TT following tumor inoculation. At ST, there was an increase in absolute number (**Figure 4J**) and a decrease in the percentage (**Figure 4K**) of CD86$^-$ mature CD4$^+$ non-plasmacytoid DCs in response to tumor, but no significant changes were observed at TT. Again we saw no changes in absolute numbers of CD86$^+$ mature CD4$^+$ non-plasmacytoid DCs at either temperature (**Figure 4L**). We did see a reduced percentage of CD86$^+$ mature CD4$^+$ non-plasmacytoid DCs at ST but not at TT following tumor inoculation (**Figure 4M**). Interestingly, despite the increased number of non-plasmacytoid DCs in mice at ST (**Figure 3G**), there are no differences in the number of mature DCs (**Figures 4F,L**) suggesting that although DC numbers appear to be increased in cold stressed mice, many of these cells are unable to become activated in the presence of a 4T1 tumor.

Due to the increased overall numbers but relatively low number of mature splenic DCs seen in mice at ST, we asked whether DCs from mice at ST were impaired at antigen presentation and their ability to activate naïve T cells. To answer this question, we performed mixed lymphocyte reactions using irradiated splenocytes from ST and TT tumor-free and tumor-bearing mice as stimulator cells and T cells from naïve ST mice as the responders. Responder and stimulator cells were co-cultured at a 2:1 ratio for 72 h and then T cell proliferation was measured by ^{3}H-thymidine incorporation. As expected, we found that stimulator cells from tumor-free mice at both ST and TT were able to induce significant T cell proliferation (**Figure 5**; tumor-free). However, stimulator cells from tumor-free mice at ST elicited significantly more T cell proliferation than those from mice at TT (**Figure 5**; tumor-free, + T cells). Interestingly, when we looked at tumor-bearing mice, we found that stimulator cells from mice at TT were able to initiate T cell proliferation while those from mice at ST were not (**Figure 5**; tumor-bearing). These results suggest that the activated DCs found in 4T1 tumor-bearing mice at TT are more efficient antigen presenting cells than DCs from tumor-bearing ST mice as demonstrated by the superior ability of TT splenocytes to elicit T cell proliferation. As the *in vitro* portion of this work was all done at 37°C, these findings also suggest that cold stress can alter DC function over a prolonged period of time after DCs are removed from the mouse.

DISCUSSION

The relationships between stress and DC function are complex and depend upon the type and duration of stress, and whether the stressor is applied *in vivo* or *in vitro*. The type of DC (i.e., isolated from the bone marrow or skin) or stage of DC maturation when a stressor is encountered may also influence the impact of a particular stress (39). Additionally, DC function is dependent on the timing of antigen exposure and/or the type of antigen used, so these factors may also affect the observed relationship between stress and DC function (44).

In addition to summarizing some of the existing data on the effects of various stressors and stress hormones on DC function, we show here that the numbers and percentages of different subsets of DCs can be dependent upon housing temperature. Since sub-thermoneutral housing temperature is the standard condition under which mice are housed throughout the world, our data suggests that only using mice which are mildly cold stressed could be limiting our full understanding of the role of DCs in immune responses, including their role in anti-tumor immunity. Specifically, we have shown that tumor-bearing mice at ST have significantly more DCs compared to tumor-bearing mice at TT. However, the increased DCs seen at ST primarily display an immature phenotype (MHC II$^-$CD86$^-$) or they up-regulate MHC II but not CD86 rendering them unable to activate CD8$^+$ T cells. The induction of signal 1 in the absence of signal 2 has been shown to lead to immune tolerance (12, 13). Thus, our studies suggest the potential for greater tolerance in mice at ST versus TT as splenocytes from ST mice were unable to activate T cell proliferation likely contributing to faster tumor growth. We observed

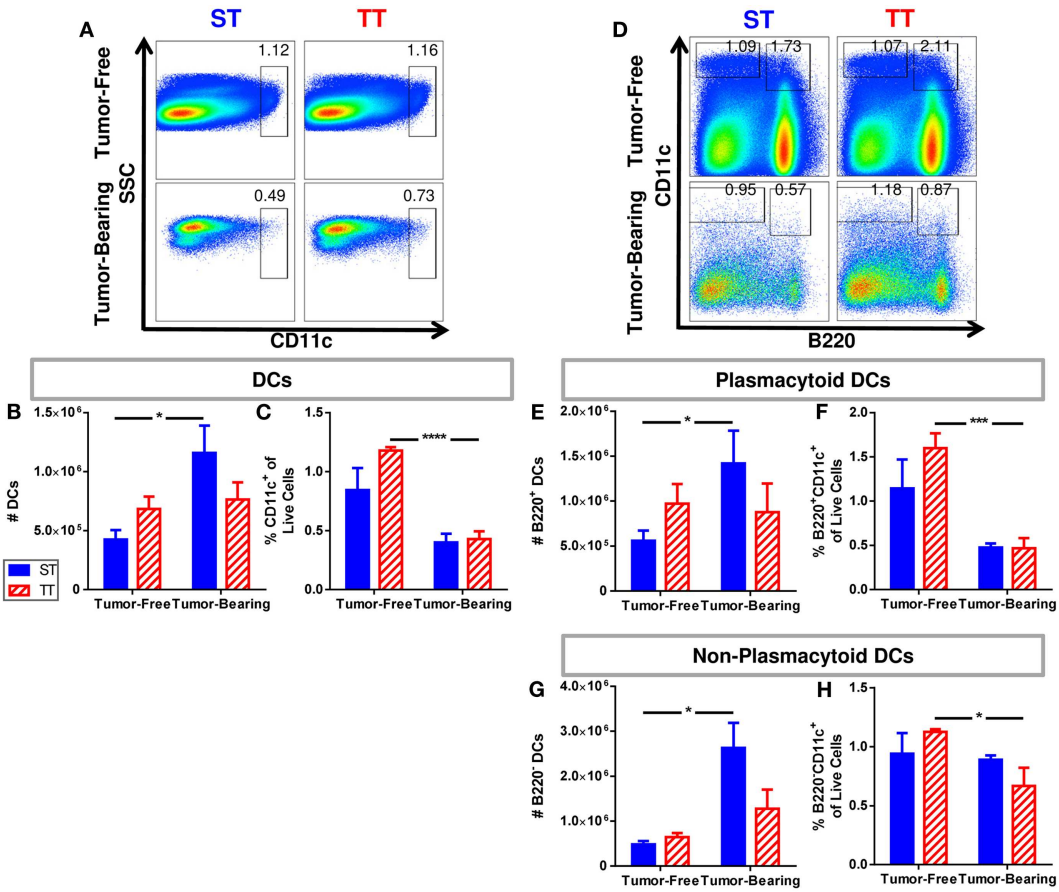

FIGURE 3 | Tumor-bearing mice maintained at ST have an increased frequency of DCs compared to those at TT. Single cell suspensions of splenocytes from 4T1 tumor-bearing mice and age-matched controls were stained for CD11c and **(D–H)** B220 and analyzed by flow cytometry. **(A)** Representative dot plots from each group show the gating strategy used to select $CD11c^+$ cells. Percentage of cells are shown above their respective gate. **(B)** The absolute number of $CD11c^+$ cells calculated from the total number of splenocytes counted in each individual mouse. **(C)** The percentage of $CD11c^+$ cells of the total population of live cells as determined by DAPI staining. **(D)** Representative dot plots from each group show the gating strategy used to select $B220^+CD11c^+$ and $B220^-CD11c^+$ cells. Percentage of cells are shown above their respective gate. **(E)** The absolute number of $B220^+CD11c^+$ cells calculated from the total number of splenocytes counted in each individual mouse. **(F)** The percentage of $B220^+CD11c^+$ cells of the total population of live cells as determined by DAPI staining. **(G)** The absolute number of $B220^-CD11c^+$ cells calculated from the total number of splenocytes counted in each individual mouse. **(H)** The percentage of $B220^-CD11c^+$ cells of the total population of live cells as determined by DAPI staining. Data presented as mean ± SEM; $n = 5$/group; Student's *t*-test; $^*p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

enhanced T cell stimulatory ability by splenocytes from tumor-free mice at ST versus TT; however, when tumors were present the ability of ST splenocytes to activate T cells was diminished. While these data presented here is limited by the fact that we used whole splenocytes instead of isolated DCs to quantify the ability of cells from mice at ST and TT to activate T cells, the results presented strongly suggest that DCs from mice under mild cold stress are less able to undergo maturation prime T cells and elicit efficient T cell responses than mice maintained under thermoneutral conditions.

One possible explanation for the differences in tumor growth in mice from ST and TT is that DCs from mice at ST are more suppressive than those from mice at TT. It has been shown that a subset of murine DCs become particularly suppressive throughout tumor growth (100–103). Our previous observations suggest that when a tumor is present, the impact of cold stress is greatly exacerbated (55). This idea is further supported by these findings showing that T cell stimulation is greatly suppressed by splenocytes from mice at ST, but not TT.

The data presented here, along with other recent publications (81, 84–90) strongly suggest that the effects of chronic mild cold stress are important to consider when working with mouse models. Moreover, when studying the impact of experimentally induced stress, such as social or psychological stress, on DCs and other immune cells, it may be important to recognize that baseline data could be significantly influenced by inherent cold stress induced by standard housing conditions for laboratory mice.

FUTURE RESEARCH QUESTIONS

New questions emerge from the data presented here with regard to the effects of stress on DCs. Most importantly, what is the mechanism by which mild cold stress influences DC function? NE is involved in activation of thermogenesis in order to increase heat

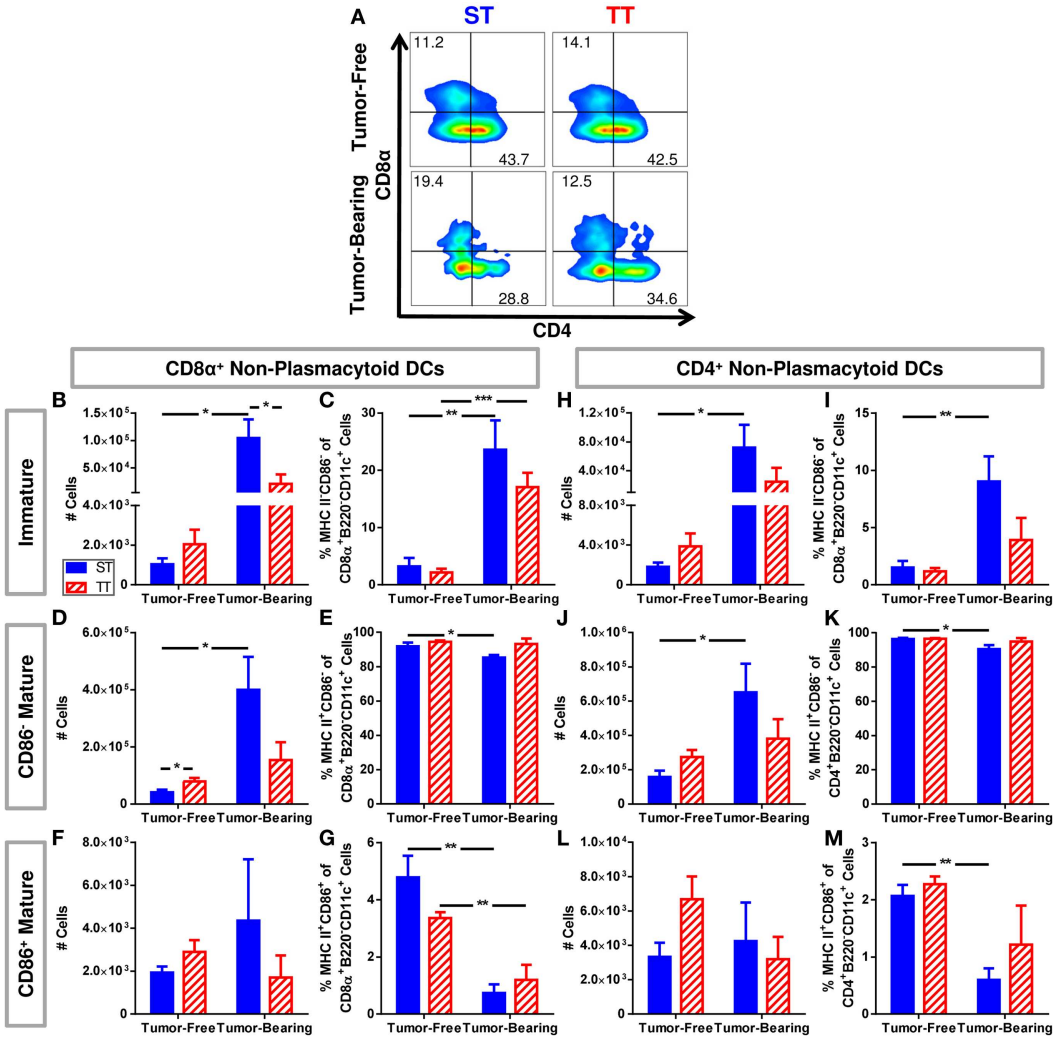

FIGURE 4 | Tumor-bearing mice maintained at ST have proportionally more non-plasmacytoid DCs than those at TT, but these DCs primarily display an immature phenotype. Single cell suspensions of splenocytes from 4T1 tumor-bearing mice and age-matched controls were stained for CD8α, CD4, CD11c, MHCII, CD86 and analyzed by flow cytometry. **(B–G)** Quantification of data describing CD8α$^+$ non-plasmacytoid DCs. **(A)** Representative dot plots from each group show the gating strategy used to select CD4 and CD8α cells from the non-plasmacytoid parent population shown in **Figure 3D**. Percentage of cells are shown above their respective gate. **(B)** The absolute number of CD8α$^+$MHC II$^-$CD86$^-$ B220$^-$CD11c$^+$ cells calculated from the total number of non-plasmacytoid cells. **(C)** The percentage of MHC II$^-$CD86$^-$ cells of the total population of CD8α$^+$ non-plasmacytoid cells. **(D)** The absolute number of CD8α$^+$MHC II$^+$CD86$^-$B220$^-$CD11c$^+$ cells calculated from the total number of non-plasmacytoid cells. **(E)** The percentage of MHC II$^+$CD86$^-$ cells of the total population of CD8α$^+$ non-plasmacytoid cells. **(F)** The absolute number of CD8α$^+$MHC II$^+$CD86$^+$B220$^-$CD11c$^+$ cells calculated from the total number of non-plasmacytoid cells. **(G)** The percentage of MHC II$^+$CD86$^+$ cells of the total population of CD8α$^+$ non-plasmacytoid cells. **(H–M)** Quantification of data describing CD4$^+$ non-plasmacytoid DCs. **(H)** The absolute number of CD4$^+$MHC II$^-$CD86$^-$B220$^-$CD11c$^+$ cells calculated from the total number of non-plasmacytoid cells. **(I)** The percentage of MHC II$^-$CD86$^-$ cells of the total population of CD4$^+$ non-plasmacytoid cells. **(J)** The absolute number of CD4$^+$MHC II$^+$CD86$^-$B220$^-$CD11c$^+$ cells calculated from the total number of non-plasmacytoid cells. **(K)** The percentage of MHC II$^+$CD86$^-$ cells of the total population of CD4$^+$ non-plasmacytoid cells. **(L)** The absolute number of CD4$^+$MHC II$^+$CD86$^+$B220$^-$CD11c$^+$ cells calculated from the total number of non-plasmacytoid cells. **(M)** The percentage of MHC II$^+$CD86$^+$ cells of the total population of CD4$^+$ non-plasmacytoid cells. Data presented as mean ± SEM; $n = 5$/group; Student's t-test; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

production to maintain normal body temperature (51, 85) and has already been strongly implicated for its roles in immunosuppression (20) and in regulating the polarization of macrophages (85). These observations strongly point to NE being a key player in the underlying relationship between cold stress and impaired DC function (55).

As mentioned earlier, cytokines affected by glucocorticoid treatment (43) or mild heating (60, 61, 63) include TNF-α, IFN-γ, IL-1α, IL-17, IL-10, and IL-12. How is the expression of these cytokines impacted by pre-existing mild cold stress in mice? In order to fully understand the impact of other types of stress in mouse models, it will be imperative to understand

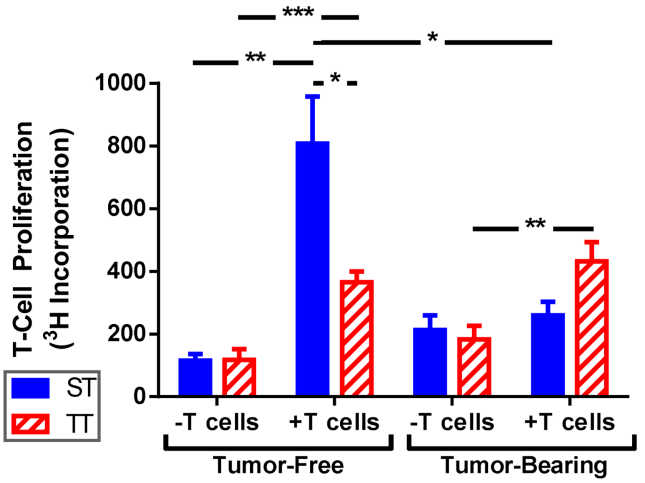

FIGURE 5 | T cells are activated better by splenocytes from mice at TT than ST. Total splenocytes from tumor-free and tumor-bearing BALB/c mice and lymphocytes from C57BL/6 mice were cultured 1:2. T cell proliferation was measured by ^{3}H-thymidine incorporation. Data presented as mean ± SEM; $n = 5$/group; Student's t-test; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

if cytokine production by DCs differs when mice are housed at sub-thermoneutrality compared to TT.

Here, we looked at DC expansion in response to inoculation with the 4T1 murine mammary carcinoma cell line. Do DCs in the presence of other tumor models respond similarly to cold stress? Similar analysis of mice at ST and TT using hematological tumors and other widely used cell lines representing different types of solid tumors, as well as human derived cell lines and patient xenograft models in immunosuppressed mice may elicit different findings regarding tumor growth control and DC function. Further, use of carcinogen-induced or transgenic mouse tumor models will all be important to establish the overall impact of cold stress on DC function.

We have shown that eliminating cold stress influences baseline properties of DCs in tumor-free and tumor-bearing mice. Therefore, a major question which should be addressed is how this may be influencing data interpretation of experiments in which additional stressors (such as social isolation) are imposed on pre-existing cold stress. It is also possible that previously demonstrated beneficial effects of mild hyperthermia on DC function could be related to the fact that control (non-heated) mice are actually cold stressed. In other words, applications of mild heat could have a similar effect on DCs as thermoneutral housing in which body temperature is not elevated. It is clear that the study of stress responses in mice should be done at more than one ambient temperature in order to understand the impact of this variable on data interpretation. Conducting experiments under thermoneutral conditions as well as sub-thermoneutral housing would help to eliminate the impact of pre-existent cold stress while studying the effects of other stressors on DC function.

In summary, since a complete understanding of DCs is critical for development of effective immunotherapies for cancer patients, it is essential to recognize that the function of these critical cells may be dependent upon ambient housing temperature and other factors which influence physiologic or metabolic stress experienced by laboratory mice used in preclinical studies.

MATERIALS AND METHODS

MICE

Female, 8–10-week-old BALB/cAnNcr (BALB/c) and C57BL/6NCr (C57BL/6) mice were purchased from the NCI (Bethesda, MD, USA). Prior to experimentation, BALB/c mice were acclimated to ST or TT for 2 weeks.

MOUSE HOUSING AT ST AND TT

Mice were maintained in specific pathogen-free facilities and were treated in accordance with the guidelines established by the IACUC at Roswell Park Cancer Institute (Buffalo, NY, USA). Cages containing Enrich-o'Cobs bedding (The Andersons, Inc., Maumee, OH, USA) housed mice 5 to a cage. Cages were held in Precision® Refrigerated Plant-Growth Incubators (Thermo Scientific; Waltham, MA, USA) maintained at 22 or 30°C. Humidity was controlled using a Top Fin® Air Pump AIR 1000 with Top Fin® airline tubing.

CELL LINE

4T1 murine mammary carcinoma cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) with 10% FBS, 10 mM L-glutamine, and 100 μg/ml penicillin/streptomycin. When cells reached ~90% confluence in culture, 1×10^4 4T1 cells were injected orthotopically into the fourth mammary fat pad of BALB/c, mice.

FLOW CYTOMETRY

Cells were collected from the spleen, tumor, and draining lymph node. Tissues were excised, washed, and filtered into a single cell suspension. Cells were counted with a hemocytometer and Trypan Blue solution. Cells were stained with Brilliant Violet 711™ anti-mouse CD4 (clone RM4-5; BioLegend; San Diego, CA, USA), Brilliant Violet 650™ anti-mouse CD3 (clone 17A2; BioLegend), Pacific Blue™ anti-mouse CDllb (clone M1/70; BioLegend), APC anti-mouse CD11c (clone N418; BioLegend), FITC anti-mouse CD86 (clone GL1; BD Pharmingen; San Jose, CA, USA), PerCp/Cy5.5 anti-mouse MHC (clone M5/114.15.2; BioLegend). Live cells were determined by staining cells with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies; Grand Island, NY, USA) and defined as DAPI-negative. Samples were analyzed on an LSRII flow cytometer (BD Pharmingen) and analyzed using FlowJo (Ashland, OR, USA) version 10.0.6.

MIXED LYMPHOCYTE REACTIONS

Spleens were excised from tumor-free and tumor-bearing BALB/c mice, and lymph nodes were excised from C57BL/6 mice. BALB/c splenocytes were irradiated at 30 Gy. BALB/c splenocytes (stimulator cells) and C57BL/6 lymphocytes (responder cells) were filtered, washed, and cultured at a ratio of one stimulator cell to two responder cells in 200 μl RPMI (10% FBS, 100 mM L-Glutamate, and 100 U/ml Penicillin–Streptomycin). After 72 h

1 μCi ^{3}H-thymidine was added for 14–18 h. T cell proliferation was determined by ^{3}H-thymidine incorporation.

DATA ANALYSIS AND STATISTICS

All data are presented as mean ± SEM All p values were determined using Student's t-tests or two-way ANOVA with Bonferroni post-tests. All statistical analysis was completed using Prism software.

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Rapid detection of dendritic cell and monocyte disorders using CD4 as a lineage marker of the human peripheral blood antigen-presenting cell compartment

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Dendritic cells (DCs) and monocytes are critical regulators and effectors of innate and adaptive immune responses. Monocyte expansion has been described in many pathological states while monocyte and DC deficiency syndromes are relatively recent additions to the catalog of human primary immunodeficiency disorders. Clinically applicable screening tests to diagnose and monitor these conditions are lacking. Conventional strategies for identifying human DCs and monocytes have been based on the use of a lineage gate to exclude lymphocytes, thus preventing simultaneous detection of DCs, monocytes, and lymphocyte subsets. Here we demonstrate that CD4 is a reliable lineage marker for the human peripheral blood antigen-presenting cell compartment that can be used to identify DCs and monocytes in parallel with lymphocytes. Based on this principle, simple modification of a standard lymphocyte phenotyping assay permits simultaneous enumeration of four lymphocyte and five DC/monocyte populations from a single sample. This approach is applicable to clinical samples and facilitates the diagnosis of DC and monocyte disorders in a wide range of clinical settings, including genetic deficiency, neoplasia, and inflammation.

Keywords: dendritic cells, monocytes, flow cytometry, immunodeficiency, humans

INTRODUCTION

Dendritic cells (DCs) and monocytes are bone marrow derived mononuclear cells involved in a wide range of immune functions. Blood DCs comprise three subsets: plasmacytoid DCs (pDCs), CD1c$^+$ myeloid DCs (mDCs), and CD141$^+$ mDCs (1–5). pDCs typically lack the myeloid antigens CD13, CD33, and CD11b and express CD123 (IL-3 receptor), CD303 [CLEC4C; Blood DC antigens (BDCA)-2], and CD304 (neuropilin; BDCA-4) (1). They are specialized to produce a rapid type I interferon response to viral infections (6). mDCs share markers in common with monocytes and granulocytes including CD13, CD33, and CD11b and perform the classical functions of DCs in taking up and presenting antigen on HLA class II molecules. DCs resembling all three subsets are found in lymph nodes (7, 8) both mDC subsets have tissue counterparts (5).

Monocytes also comprise a number of distinct functional subsets delineated by expression of CD14 and CD16 in humans. CD14$^+$ CD16$^-$ "classical monocytes" perform inflammatory functions including phagocytosis, production of reactive oxygen species, nitric oxide, and TNFα (9). Two additional populations have been described: CD16$^+$ CD14^{low} "non-classical" monocytes and CD14$^+$ CD16$^+$ "intermediate" monocytes (3, 10). There is variation in how these cells are divided, with a position paper on nomenclature suggesting that intermediate monocytes may be grouped with non-classical monocytes (both linked by the expression of CD16) while more recent gene expression studies suggest that intermediate monocytes are more closely linked to classical monocytes (11). Both by flow cytometry and gene set enrichment analysis, intermediate monocytes appear to be part of a continuum (12). It is clear, however, that the non-classical pole of the spectrum contains cells with higher class II expression, allostimulatory capacity, and cytokine production that have led to their classification as a type of DC (2, 13). CD16$^+$ non-classical monocytes are also smaller and become closely associated with the endothelium upon adoptive transfer into mice (11). A wide range of studies show that non-classical monocytes are increased by exercise, autoimmune disease, bacterial sepsis, tuberculosis, and HIV infection, reviewed in Ref. (10).

Routine analysis of human blood DCs and monocytes is usually confined to the enumeration of classical monocytes by automated blood counters. The complexity of changes in DC and monocyte subsets is not visible in most clinical scenarios. Flow cytometry is frequently used to analyze lymphocyte subsets but simultaneous detection of DCs and monocytes is hampered by the lack of a positive lineage marker. Although robust platforms for DC counting have been described, these invariably depend upon identifying MHC class II (HLA-DR) expression by lineage (lin) negative cells, a population defined by the exclusion of T cells (CD3), B cells (CD19, CD20), NK cells (CD56), monocytes (CD14, CD16), and progenitors (CD34) (2, 14, 15). The exclusion of lin$^+$ lymphocytes and monocytes either precludes simultaneous measurement with DCs or demands large number of fluorescence channels (4, 15). Differential DC and monocyte counting is therefore rarely performed as a clinical test.

Blood DC antigens 1–4 are helpful for identifying human DC subsets (1) but are often used to define pDC and mDC subsets within the HLA-DR$^+$ lin$^-$ population (4, 16). This is especially true for CD1c (BDCA-1), which identifies the main population of mDCs but is also expressed on B cells (1). CD303 (BDCA-2; CLEC4C) and CD304 (BDCA-4; neuropilin) are relatively robust markers for pDCs and give reliable counting even from unselected peripheral blood mononuclear cells. CD141 (BDCA-3) defines a very small population of mDCs (1, 2).

Early reports demonstrated that DCs and monocytes both express CD4, but at lower levels than T helper cells (17–19). While the CD4 glycoprotein acts as a co-receptor for the T cell receptor on T helper cells, its role on myeloid cells is less clear. CD4 also functions as a receptor for IL-16 (lymphocyte chemoattractant factor), which is capable of both recruiting CD4$^+$ immune cells and driving cell growth (20). Studies in HIV suggest that CD4 may augment Fc receptor signaling (21). Numerous studies report the presence of CD4 on myeloid hematopoietic precursors including myeloid malignancies (22). While in mice, expression of CD4 is retained by T lymphocyte and myeloid precursors but lost by B lymphocyte precursors (23), the function and fate of CD4 expressing precursors in humans has not been adequately mapped.

Here we show that CD4 has significant utility as positive lineage marker of human blood antigen-presenting cells, allowing delineation of all the currently recognized subsets of human blood monocytes and DCs. This allowed us to adapt a standard 6-color flow cytometry protocol for lymphocyte immunophenotyping to allow simultaneous quantification of DC and monocyte subsets.

MATERIALS AND METHODS

PARTICIPANTS

For assay development, blood was collected into EDTA from 33 patients and 5 healthy controls. Patients were randomly selected from the regional clinical immunology laboratory. Patients were aged 2–83 (median 44) and had unknown conditions for further investigation [11], immunodeficiency [7], autoimmunity [7], or atopy [3]. To test the assay in pathological states, fresh blood was obtained from a 33-year old man with DCML deficiency due to GATA-2 deficiency and a 77-year old man with blastic plasmacytoid dendritic cell neoplasm (BPDCN). To recapitulate septic conditions with left-shifted myelopoiesis, eight samples of cryopreserved G-CSF-mobilized peripheral blood stem cells (PBSC) were thawed and washed for analysis. Ethical approval was granted by Newcastle and North Tyneside Research Ethics Committee 1.

PROCESSING AND ANALYSIS

TruCount™ tubes (Becton Dickinson; BD) containing a defined number of polyfluorescent beads per test were used in a single step "lyse-no wash" method to enumerate blood cells, according to the manufacturer's instructions. Fifty microliters of blood was transferred directly to the TruCount™ tube. Antibodies were added directly and staining was performed at 4°C for 20 min. Red cell lysis was achieved by addition of 450 μl of the proprietary reagent at room temperature for 10 min. Samples were then directly analyzed by flow cytometry and the number of cells per microliters of blood was calculated from the number of events and the fraction of beads analyzed, according to the formula:

$$\text{Cells}/\mu\text{l of population x} = \left(\text{population x events}/\text{bead events}\right) \times \left(\text{beads per test}/50\right)$$

The TBNK reagent (BD catalog number 337166) contains six reagents as described in **Table 1** for the detection of CD45, CD3, CD4, CD8, CD19, and CD16/56. Combined with TruCount tubes this reagent allows the enumeration of CD4$^+$ and CD8$^+$ T cells, B cells, and NK cells. The modification for detecting DCs and monocytes was to add CD14-APC-Cy7 to the CD8 channel and CD304-APC to the CD19 channel.

Flow cytometry was performed on FACSCanto two or three laser instruments running DiVa version 6 (BD). Instrument PMTs were set using whole blood stained with single antibodies. FSC and SSC thresholds were set at zero to in order to capture TruCount™ bead events. A threshold was set on CD45, allowing detection of leukocytes, but discounting platelet and red cell debris. Thresholds were 1000 on 635 780/60 channel for the 8-color panel and 1000 on 488 760LP channel for the 6-color panel. Isotype controls were performed for all antibodies at the initial set up and are shown specifically for CD4. Automatic compensation was performed using compensation beads (BD). Approximately 200,000 CD45$^+$ events were acquired per sample (**Table 2**). Analysis was performed with FlowJo version 9.5.2 (TreeStar). Graphing and statistical analysis were performed with Prism version 6 (GraphPad).

Table 1 | Antibody panels used for flow cytometry analysis.

Fluorochrome	8-Color DC panel	Modified 6-color panel
FITC	CD3 [a](345763; BD) CD19 (345776; BD) CD20 (345792; BD) CD56 (345811; BD)	CD3
PE	CD16 (555407; BD)	CD16, CD56
PERCPCy5.5	CD123 (558714; BD)	CD45
PECy7	CD14 (557742; BD)	CD4
APC	CD141 (130-090-907; Miltenyi)	CD19 **CD304** (130-090-900; Miltenyi)
APCCy7	CD14 (557742; BD)	CD8 **CD14** (557831; BD)
V450	CD11c (560369; BD)	
V500	HLA-DR (561224; BD)	

[a] *(Catalog number; supplier).*

CD4 V500 (560768; BD), CD4 PE (555347; BD), CD3 PE (345765; BD), CD19 PE (555413; BD), and CD56 PE (345812; BD) were used in additional experiments. Bold text indicates the antibodies added to modify the 6-colour TBNK(TM) panel (337166; BD).

Table 2 | Flow cytometry events recorded for analysis of monocyte and DC subsets.

Cell population	8-Color DC panel events collected median (IQ range)	Modified 6-color panel events collected median (IQ range)
DC PANEL COMPARISON (*N* = 24)		
Total	204634 (125021)	197925 (101726)
CD14 monocytes	24422 (13512)	9289 (5981)
CD16 monocytes	1590 (2973)	570 (934)
CD14 CD16 monocytes	1381 (1443)	633 (499)
DC	971 (697)	369 (327)
DC SUBSET DISCRIMINATION (*N* = 14)		
Total	112172 (39906)	130255 (116775)
mDC	408 (218)	239 (98)
pDC	154 (295)	81 (190)

RESULTS

CD4 IS A LINEAGE MARKER FOR BLOOD ANTIGEN-PRESENTING CELLS

We designed a flow cytometry panel to examine known subsets of monocytes and DCs. Working with whole blood, the standard approach of lineage exclusion and HLA-DR selection required a minimum of eight fluorescence channels to categorize all monocyte and DC subsets. Throughout the study we used this 8-color panel as the standard reference panel for DC and monocyte enumeration (**Figure 1A**). In the $CD45^+$ SSC^{low} gate, we first gated on HLA-DR^+ lineage negative cells (CD3, CD19, CD20, CD56) to obtain all the monocyte and blood DC populations. Monocytes were analyzed by CD14 vs. CD16 and the double negative cells further separated into pDCs and two subsets of mDCs. We then explored the expression of CD4 on different leukocytes. We used specific fluorophores to identify each lineage-positive fraction and the schema outlined in **Figure 1A** to identify DCs and monocytes. $CD4^+$ T cells are seen in the $CD3^+$ fraction but B cells and NK cells are CD4 negative (**Figure 1B**). Back-gating illustrates where each population lies on the HLA-DR vs. lineage plot. This indicates that NK cells are most likely to broach the lin-DR^+ gate. As many NK cells express CD16, the gate must be placed sufficiently low on the lineage axis to prevent subsequent NK contamination of the CD16 monocyte gate. All monocyte and DC populations express CD4 above isotype control (**Figures 1C,D**). Back-gating shows the relative HLA-DR expression of these populations. The position of the HLA-DR vs. lineage gate is critical as some monocytes express low HLA-DR. The lower border of the gate on the HLA-DR axis was judged according to isotype controls for HLA-DR (not shown) and the inclusion of a maximum number of $CD14^+$ monocytes, the cells with the lowest HLA-DR expression. To ensure that all monocytes and DCs were being captured by this strategy, the gate was also drawn simply as a lineage negative gate (Figure S1 in Supplementary Material). Although this results in the inclusion of HLA-DR negative myeloid precursors and basophils (4), these are subsequently excluded out by the down-stream gating on monocytes and DCs. This at least confirms that no cells are lost by the HLA-DR^+ lineage negative gate as it was defined in **Figure 1A**.

CD4 EXPRESSION IDENTIFIES MONOCYTES AND DCs WITHIN A LYMPHOCYTE PHENOTYPING PANEL

Having established the expression of CD4 by different leukocytes, we explored the ability of this marker to capture DCs and monocytes. The previous results predicted that a 2D plot of CD4 vs. CD3 should display a population of $CD4^+$ $CD3^-$ cells containing DCs and monocytes, with slightly lower CD4 expression than $CD4^+$ T cells. Both these markers are found within a standard lymphocyte phenotyping panel, offering the potential to enumerate DCs monocytes and lymphocytes simultaneously. We used the TBNK™ reagent (BD) containing six colors to detect T, B, and NK lymphocytes and provide CD4:CD8 ratios from a single tube. As with the previous panel, mononuclear cells are initially separated from granulocytes, beads and debris by FSC and SSC properties (**Figure 2A**) and $CD45^+$ cells selected (not shown). The subsequent 2D plot of CD4 vs. CD3 shows the predicted population of $CD4^+$ $CD3^-$ cells (**Figure 2B**) that does not contain B cells (**Figure 2C**) or NK cells (**Figure 2D**).

To characterize these cells further, we modified the TBNK reagent by re-using fluorescence channels that had already been excluded by the gating, namely APC (conjugated to CD19) and APCCy7 (conjugated to CD8). CD14 APCCy7 in combination with CD16/56 thus allows resolution of monocyte subsets and the $CD14^-$ $CD16^-$ parameter space, containing DCs (**Figure 2E**). Note that the $CD4^+$ $CD16^+$ population identified in **Figure 2D** is confirmed as the $CD16^+$ non-classical monocyte by low to medium expression of CD14. Further display of the $CD14^-$ $CD16^-$ population by CD4 vs. CD304-APC enabled the assay to differentiate between mDCs and pDCs (**Figures 2F,G**). This also confirms that pDCs have the highest CD4 expression among DCs and monocytes. The TBNK reagent and its modification are detailed in **Table 1**.

THE MODIFIED TBNK PANEL PROVIDES ACCURATE ENUMERATION OF MONOCYTES AND DCs

To provide a proof-of-concept that modification of the 6-color TBNK reagent with CD14 APCCy7 could reproduce the enumeration of DCs and monocytes, we ran 24 samples in parallel with the standard 8-color panel (using HLA-DR vs. lineage as an initial gate). A further 14 samples were run including both CD14 APCCy7 and CD304 APC with the TBNK reagent to evaluate the ability to split DCs into pDCs and mDCs. To reproduce the conditions of a clinical test as closely as possible, we took samples referred to the regional clinical immunology lab and prepared and analyzed them independently on separate machines.

Both panels were run using TruCount tubes to achieve absolute quantification per microliters of blood. Results from the modified TBNK reagent were highly correlated with those of the standard 8-color panel. The least correlated results were produced for total DCs and mDCs. This is probably due to the fact that the 6-color strategy did not include a positive marker of mDCs (except for CD4); they were defined by the exclusion of other populations so it is possible for a variable low level of contaminating cells to be included. Approximately 200,000 total events were recorded in order to collect at least 100 events for rare subsets of DCs (**Table 2**).

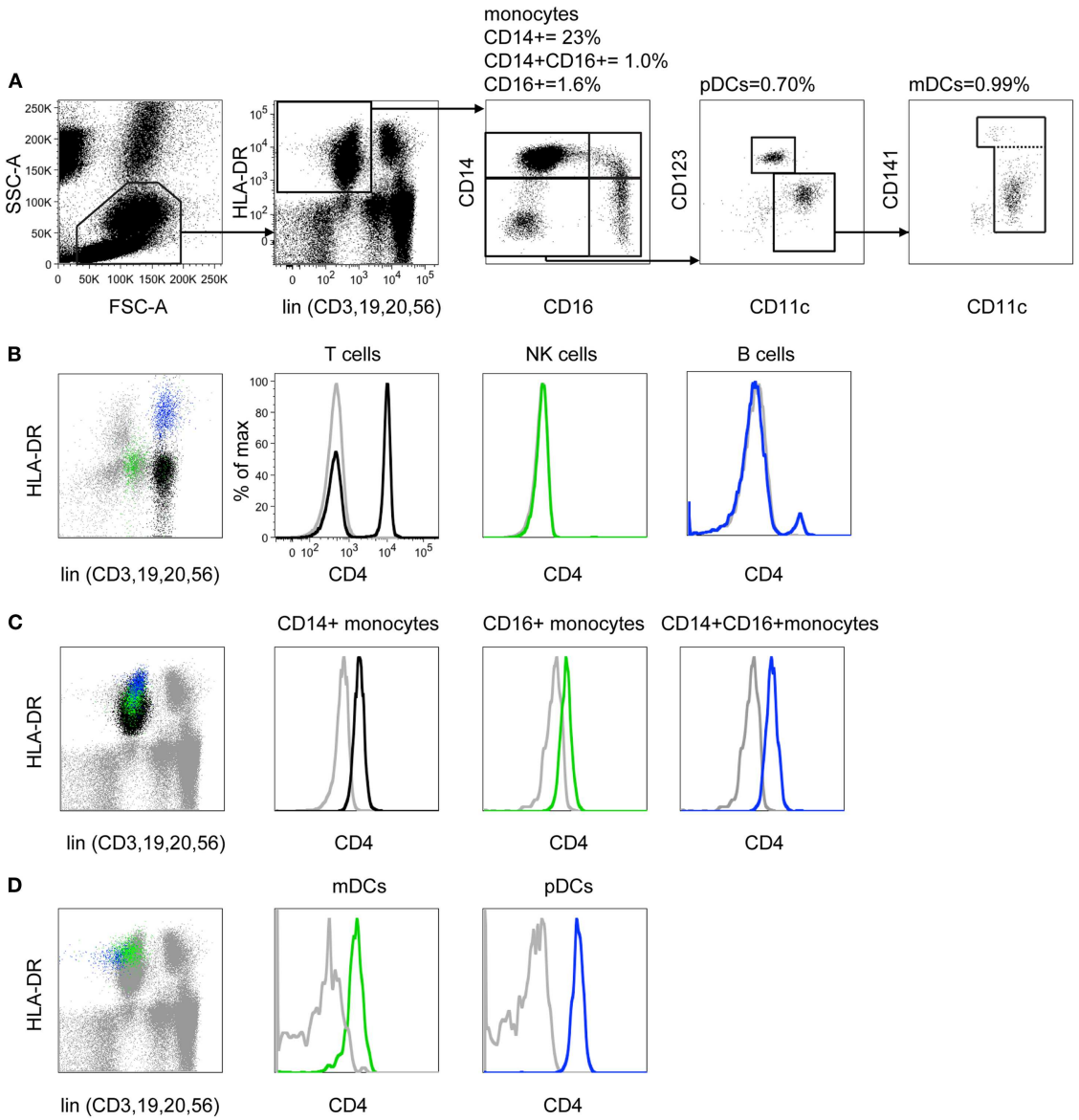

FIGURE 1 | CD4 is differentially expressed on monocytes and DCs. (A) In an 8-color DC profiling panel FSC and SSC parameters are used to distinguish mononuclear cells from granulocytes, counting beads, and debris. Mononuclear cells are confirmed as $CD45^+$ SSC low (gate not shown). Lineage markers (CD3, 19, 20, 56) are used to remove T, B, and NK cells from analysis and DR^+ monocytes and DCs are selected. The CD14 vs. CD16 plot reveals three subsets of monocytes and $CD14^-$ $CD16^-$ DCs. Plasmacytoid DCs are defined as $CD11c^{low}$ $CD123^+$. Two subsets of CD11c myeloid DC are split by CD141. Percentage values shown indicate the proportion of gated cells relative to $CD45^+$ SSC low cells. **(B)** $CD3^+$ T cells (black), $CD56^+$ NK cells (green), and $CD19^+$ B cells (blue) are back-gated onto the lineage vs. DR plot to demonstrate the locations of these populations. Expression of CD4 was then tested (colored histograms) relative to isotype control (gray). Note that a small population of activated DR^+ $CD4^+$ T cells overlaps the B cell population. **(C)** $CD14^+$ monocytes (black), $CD16^+$ monocytes (green), and $CD14^+16^+$ monocytes (blue) are back-gated onto the lineage vs. DR plot. Expression of CD4 is shown (colored histograms), relative to isotype control (gray). **(D)** Myeloid DC (green) and plasmacytoid DC (blue) are back-gated onto the lineage vs. DR plot. Expression of CD4 is shown (colored histograms), relative to isotype control.

DETECTION OF DC AND MONOCYTE DISORDERS IN CLINICAL SPECIMENS

To further test the clinical utility of the modified TBNK reagent, we selected two cases where highly abnormal numbers of DC and monocytes were detected using the 8-color panel and re-examined them with the modified TBNK test. The results are plotted on **Figure 3**.

Case 1

A 33-year-old man presented to rheumatology with a history of pyogenic infections, chronic papillomatosis, and recurrent erythema nodosum. Automated blood counts were normal except for a monocyte count 0.03×10^9/l. DC, monocyte B, and NK lymphoid (DCML) deficiency was suspected and GATA-2 mutation subsequently confirmed by sequencing (24). A blood sample

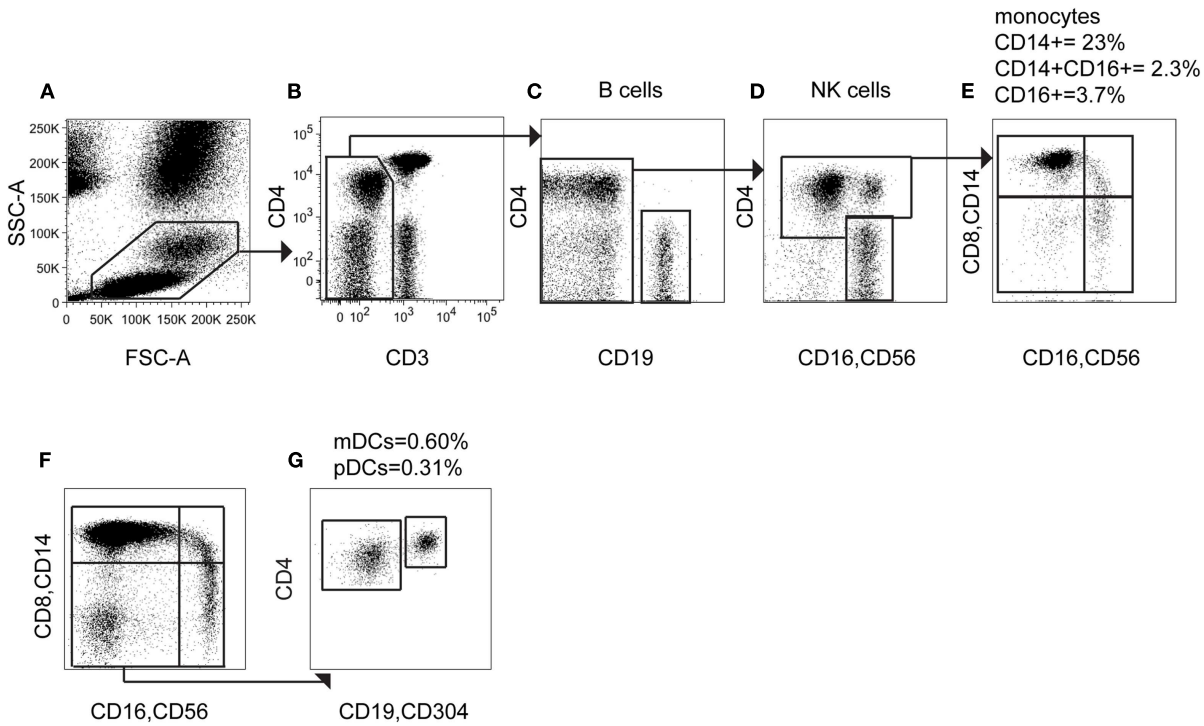

FIGURE 2 | Using CD4 as a lineage marker allows monocytes and DCs to be counted with a 6-color immunophenotyping panel. **(A)** In a 6-color immunophenotyping panel modified by addition of CD14 antibody, mononuclear cells are gated based on FSC and SSC parameters. **(B)** CD3 negative cells, including DCs and monocytes, B cells and NK cells are gated out from T cells. **(C)** $CD19^+$ B cells are removed from the $CD3^-$ population. **(D)** $CD16^+$ $CD56^+$ NK cells are removed from analysis and the $CD4^+$ population selected. **(E)** Visualized on a CD14 vs. CD16 plot, the $CD4^+$ population contains three subsets of monocytes and $CD14^-$ $CD16^-$ DC. **(F)** Adding CD304 antibody to the assay allows further separation of the $CD14^-$ $CD16^-$ DC population into **(G)** $CD4^+$ $CD304^-$ mDCs and $CD4^{bright}$ $CD304^+$ pDCs.

obtained during routine clinical monitoring was analyzed with the modified TBNK panel (**Figure 3C**). Significant and parallel depletion of all monocyte and DC subsets was observed by both methods.

Case 2

A 77-year-old man presented to dermatology with progressive skin nodules and plaques. Immunohistochemistry of a skin biopsy revealed a dense infiltrate of mononuclear cells positive for CD4, CD56, and CD123 consistent with a diagnosis of BPDCN. The leukemic expansion of pDCs was evident with both 8-color and modified TBNK (**Figure 3D**) tests. In addition, a relative expansion of intermediate and non-classical monocytes was also found by both methods. In the pDC analysis, the 8-color method appeared to underestimate; we attribute this to the expression of CD56 by BPDCN which caused some cells to be lost from the HLA-DR^+ $lineage^-$ gate as they shifted into the lineage+ fraction. In this case, using CD4 as a positive DC marker proved to be a more reliable approach.

ENUMERATION OF DCs AND MONOCYTES IN INFLAMMATORY STATES

Septic shock and other inflammatory conditions are associated with an expansion of monocytes bearing lower HLA-DR. We therefore explored whether detecting monocytes and DCs by CD4 expression would circumvent the problem of counting cells with low HLA-DR expression in an HLA-DR^+ $lineage^-$ gate, or be subject to similar pitfalls. As a model of septic shock we took samples of G-CSF mobilized peripheral blood cells, which also show expanded monocytes with low HLA-DR, and compared counts derived from the two panels. The HLA-DR^{low} fraction of the DC/monocyte gate also expressed lower CD4 (**Figures 4A,B**) but as with the HLA-DR^+ $lineage^-$ gating approach (Figure S1 in Supplementary Material), it was possible to extend the lower limit of the gate to include the cells with lower expression of CD4 (**Figure 4C**) resulting in positively correlated results between the two methods (**Figure 4D**).

DISCUSSION

In this study we show that CD4 expression may be used within a lymphocyte typing panel to identify DCs and monocytes and that this has clinical utility in enabling the simultaneous detection of nine subsets of lymphocytes, monocytes, and DCs with only six flow cytometry channels.

Dendritic cells and monocytes have been known to express CD4 since the early descriptions of blood DCs and from studies of infectivity by HIV (17–19) but recent strategies for analysis of DCs and monocytes have typically relied upon resolving HLA-DR^+ $lineage^-$ cells (3, 4). CD4 has been used in combination with a lineage cocktail to identify DCs but lymphocytes are still excluded by this approach (16). The use of CD4 as a positive lineage

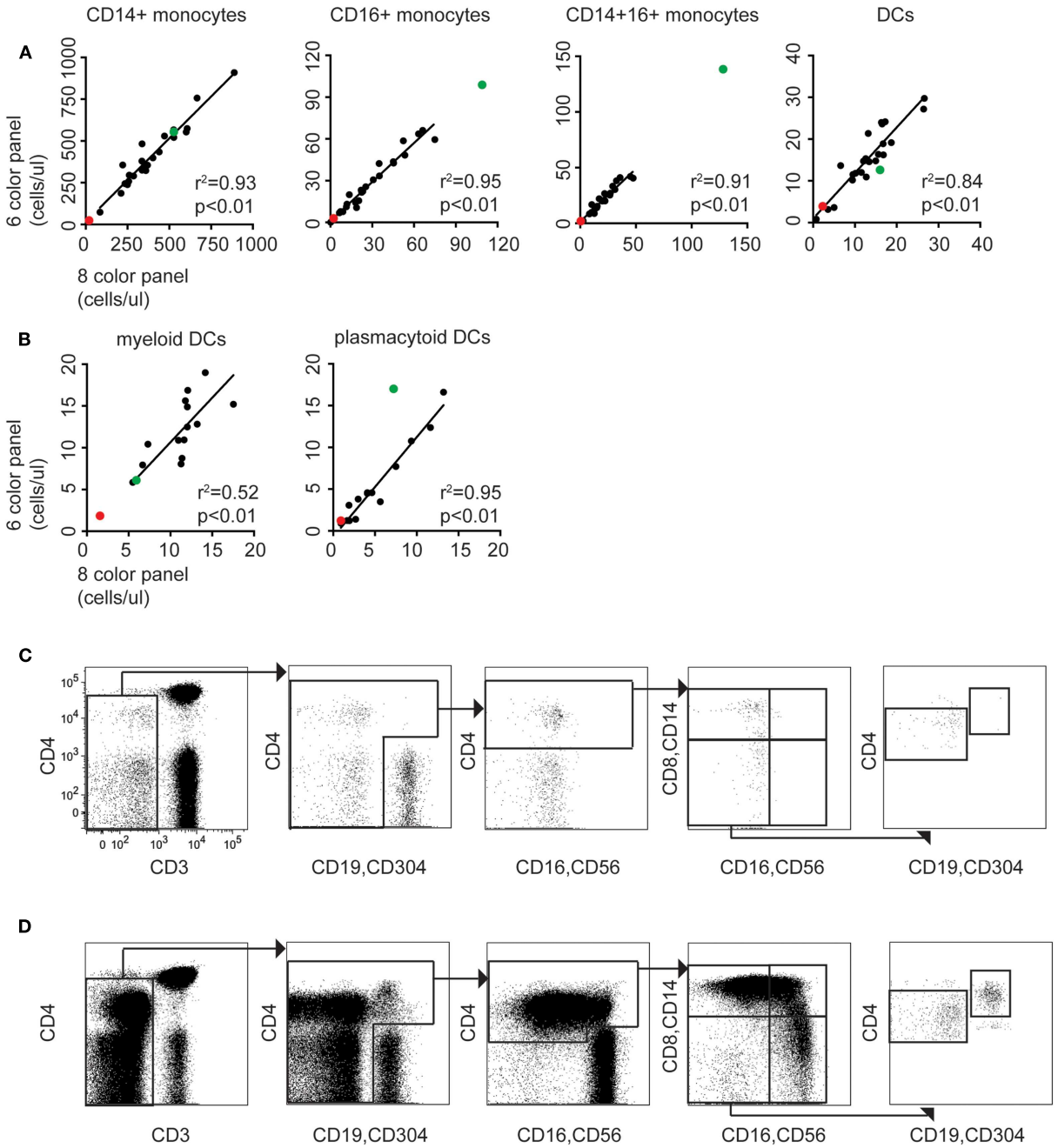

FIGURE 3 | The modified TBNK assay allows rapid quantitation of APC subsets, consistent with conventional methods. **(A)** Correlation between monocyte and DC counting performed using the 6-color immunophenotyping panel modified by addition of CD14 vs. the standard 8-color DC panel ($n = 24$). Red dots indicate results for case 1 (DCML deficiency). Green dots indicate case 2 (BPDCN). Linear regressions, r^2 and p values stated on the plots exclude these outlier cases. **(B)** Correlation between mDC and pDC counting using the 6-color panel modified with CD14 and CD304 vs. the standard 8-color DC panel ($n = 14$). **(C)** The modified TBNK assay applied to case 1 (DCML deficiency). Note the scarcity of $CD3^-$ cells relative to $CD3^+$ T cells. This arises from reduction in B cells, absence of NK cells, and virtual absence of all monocyte and DC subsets. The small population of $CD3^-$ $CD4^{bright}$ cells gated out in the initial plot are activated T cells with reduced CD3 expression. **(D)** The modified TBNK assay applied to case 2 (BPDCN).

marker, in combination with CD3, preserves the identification of lymphocyte subsets. Multiplexing fluorescence channels with more than one antibody appears to be a reliable means of expanding the number of parameters. Although we cannot exclude that aberrant expression of surface markers in some conditions may distort the analysis, this is true of any flow cytometry assay and our modified 6-color strategy actually enhanced the detection of the malignant $CD4^+$ $CD56^+$ pDC clone compared with an HLA-DR

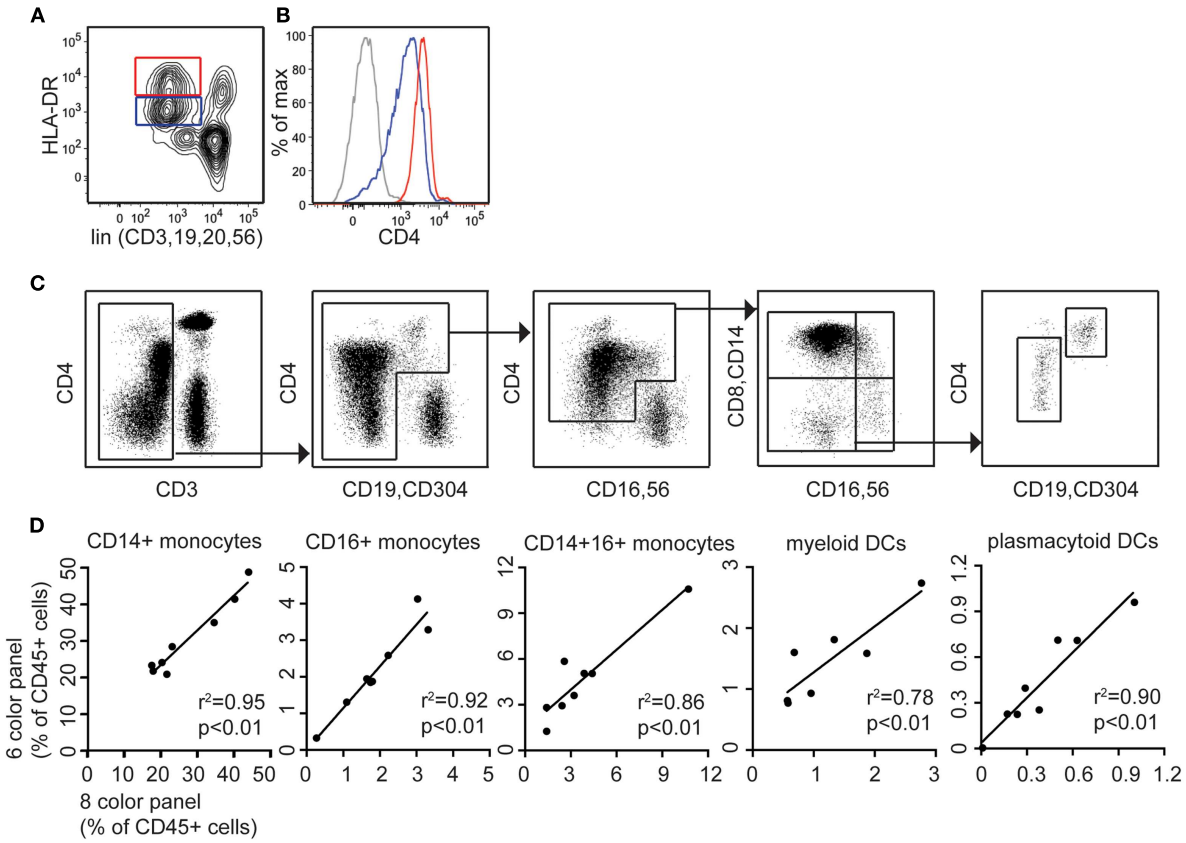

FIGURE 4 | CD4 is expressed on both DR low and DR bright populations following G-CSF mobilization. The modified TBNK assay allows clear discrimination of lymphocyte, monocyte, and DC populations in left-shifted blood. **(A)** The lineage DR plot used for 8-color DC profiling shows DR^{bright} and DR^{low} populations of monocytes and DCs in G-CSF mobilized blood. **(B)** CD4 expression is highest on lin^- DR^{bright} cells but also positive on lin^- DR^{low} cells. **(C)** The modified TBNK assay applied to G-CSF mobilized peripheral blood stem cells (PBSC) permits effective discrimination of lymphocyte, monocyte, and DC populations. **(D)** Correlation between 8-color and modified TBNK methods for DCs and monocytes in PBSC.

vs. lineage approach. Employing a single standard mononuclear cell profiling panel and multiplexing antibodies is likely to prove especially beneficial where resources and flow cytometry parameters are restricted, as in many clinical service laboratories. Here we provide proof-of-concept but additional validation and generation of local normal ranges will be required for clinical use.

This type of assay will be useful for identifying DC deficiency as recently described in a number of novel human syndromes (24–26). A typical approach to identifying immunodeficiency uses screening investigations to refine the differential diagnosis followed by more specialist investigations to specify the defect (27). The capacity to enumerate monocytes and DCs as part of an existing lymphocyte immunophenotyping platform, is an important addition to the screening repertoire. Between the 2009 and 2011 revision of the IUIS Primary Immunodeficiency Classification, 7 of the 15 new disease entities concerned the myeloid antigen-presenting cell compartment (28). The development of means to rapidly enumerate DCs and monocytes is therefore an unmet need of clinical importance (29).

Expansion of specific monocyte subsets has been observed in infection and inflammation in many clinical contexts. The delineation of monocytes is not always straightforward in relation to other cells such as NK cells through the expression of CD56 (30) or owing to the loss of HLA-DR expression during neoplasia or inflammation (31, 32). HLA^- DR^{low} monocytes also express less CD4. Rigorous comparison of gating strategies has shown that all monocytes may be collected using only HLA-DR, CD14, and CD16 (32); here we show that a CD4 gate with a lower threshold also captures the same events.

Bringing blood DC and monocyte subset analysis into routine clinical practice will most likely reveal even greater variety and subtlety of DC and monocyte deficiency states. New correlations between human disease and the behavior of this compartment are anticipated.

AUTHOR CONTRIBUTIONS

Laura Jardine performed experiments, analyzed data, and wrote the manuscript. Dawn Barge designed the study, performed experiments, and analyzed data. Ashley Ames-Draycott performed experiments, Sarah Pagan performed experiments. Sharon Cookson performed experiments. Gavin Spickett commented on the manuscript. Muzlifah Haniffa commented on the manuscript. Matthew Collin designed the study and wrote the manuscript. Venetia Bigley designed the study and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2013.00495/abstract

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Dendritic cell subsets in type 1 diabetes: friend or foe?

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Type 1 diabetes (T1D) is a T cell mediated autoimmune disease characterized by immune mediated destruction of the insulin-producing β cells in the islets of Langerhans. Dendritic cells (DC) have been implicated in the pathogenesis of T1D and are also used as immunotherapeutic agents. Plasmacytoid (p)DC have been shown to have both protective and pathogenic effects and a newly described merocytic DC population has been shown to break tolerance in the mouse model of T1D, the non-obese diabetic (NOD) mouse. We have used DC populations to prevent the onset of T1D in NOD mice and clinical trials of DC therapy in T1D diabetes have been initiated. In this review we will critically examine the recent published literature on the role of DC subsets in the induction and regulation of the autoimmune response in T1D.

Keywords: dendritic cells, type 1 diabetes, T regulatory cells, autoimmunity, tolerance

INTRODUCTION

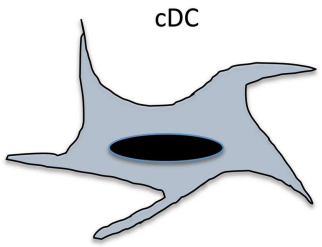
Type 1 diabetes (T1D) is an autoimmune disease characterized by immune mediated destruction of the insulin-producing β cells in the islets of Langerhans of the pancreas. The pathogenesis of T1D is multifactorial with genetic, immunological, metabolic, and environmental factors all contributing (1). It begins with a loss of self-tolerance to islet-derived self-antigens, which usually occurs early in life. This could occur as a result of a viral infection targeting the pancreas or following pancreatic remodeling. These insults lead to the death of β cells, release of self-antigens, and induction of inflammatory cytokines such as TNF-α and IL-1-β. Dendritic cells (DC) present within the pancreas take up released β-cell-derived antigens and migrate to the draining lymph nodes (LN) and activate naïve islet-specific CD4 and CD8 T cells. Depending on the signals delivered to the T cell by DCs the T cells will differentiate into either inflammatory effector cells such as T helper (Th)1 cells or anti-inflammatory Th2 or regulatory (Treg) cells. Activated islet-specific T cells then migrate to the pancreas where they infiltrate and collect around the islets. The early infiltrates appear to be dominated by Th2 and Treg cells; this is termed peri-insulitis as there is little invasion into the islet. At some point, the infiltrate becomes invasive and begins to enter and destroy the β cells, and the balance between the regulatory and inflammatory T cell populations is lost. The precise factors that trigger both loss of self-tolerance and the development of invasive insulitis are not well understood. DCs play important roles at all stages of the autoimmune response in T1D due to their pivotal role in activating naïve T cells and in maintaining self-tolerance (2). This review will explore recent developments in our understanding of the roles DC play in the pathogenesis, and prevention, of T1D (**Figure 1**) as well as the therapeutic potential of DCs for the prevention and treatment of T1D. Much of the work in this area has been focused on the non-obese diabetic (NOD) model of the disease. This mouse strain spontaneously develops diabetes and shares many of the genetic and immunological features of the human disease (3). Where possible we will highlight similarities and differences between NOD mouse studies and relevant studies in human T1D patients.

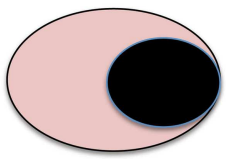
DENDRITIC CELL SUBSETS

Since their discovery by Ralph Steinman in the 1970s (4–6) DCs have been the subject of intense study. DCs are antigen presenting cells (APC) that bridge the innate and adaptive immune systems (7). They act as sentinels of the immune system through their location in peripheral tissues, where they form a dense network, and their ability to respond to pathogens through expression of pathogen recognition receptors such as Toll-like receptors (TLR). While in peripheral tissues DCs have an immature phenotype characterized by low levels of major histocompatibility complex (MHC) and co-stimulatory molecules (CD80, CD86) and a high endocytic rate; the function of tissue DCs is the detection and processing of antigen. Upon interaction with a pathogen DCs become activated, undergo maturation and migrate to the draining LN. This maturation leads to an increase in the expression of MHC, CD80, and CD86, a decrease in endocytosis and a change in the pattern of chemokine receptor expression. Mature DCs entering the T cell zone of the draining lymph node are primed to present antigen to naïve T cells. Even in the absence of pathogen there is a steady state migration of DC from the periphery to the LN and this is necessary for the maintenance of peripheral tolerance (8).

DC PHENOTYPE

There are two main classes of DC: plasmacytoid DCs (pDCs) and conventional or classical DCs (cDCs) (7). pDCs are primarily located in the blood and lymphoid organs and produce high levels of type 1 interferon (IFN) following engagement of TLRs with foreign nucleic acids (9). pDCs express a restricted set of TLRs, TLR7, and TLR9, that recognize foreign RNA and DNA respectively as well as several unique markers that distinguish them from cDCs. These include BDCA-2 and ILT7 for human pDCs and SiglecH and Bst2 for murine pDCs. In the mouse pDCs are commonly identified as $CD11c^{low}$ $B220^{+}$ $SiglecH^{+}$ $Bst2^{+}$. Human pDCs are $CD11c^{-}$ and in addition to BDCA-2 and ILT7 also express CD123 and BDCA-4 (9). There are two main populations of cDCs in mouse: $CD11c^{+}$ $CD11b^{-}$ $CD8\alpha^{+}$ $Clec9A^{+}$ and $CD11c^{+}$ $CD11b^{+}$ $CD8\alpha^{-}$ $Clec9A^{-}$ commonly referred to as CD8 DC and CD11b





PROTECTION

Protective subsets:
- CD8 DC – migrate to PLN and maintain tolerance

Mechanisms of protection:
- Induction of Treg
- PD1-PDL-1 interaction with T cells
- Interaction with tTreg to control migration

Mechanisms of protection:
- Produce IDO, TGF, IL-10 in response to iNKT
- Induction of Treg
- Local production of type 1 IFN in pancreas may be protective

DISEASE

Disease inducing subsets:
- CD11b DC – accumulate in inflamed islets
- mcDC – present islet antigens

Mechanisms of disease induction:
- Increased IL-12 production
- Cross-present Ag to CD8 T cells
- TNF, IL-1 induce DC maturation

Mechanisms of disease induction:
- Produce type 1 interferon in response to viral infection – in cooperation with B1 B cells and neutrophils

FIGURE 1 | Summary of the roles of cDC and pDC in the prevention and induction of T1D.

cDC respectively. Both CD8 and CD11b cDC arise from a common precursor characterized by expression of the C-type lectin receptor DNGR-1, but it appears that pDC arise from a distinct precursor as yet to be defined (10, 11). Recently the CD11b cDC population has been further subdivided on the basis of endothelial cell selective adhesion molecule (ESAM) expression (12). Similar populations can be identified in human such that $CD11c^+$ $Clec9A^+$ $BDCA\text{-}3^+$ DCs correspond to CD8 cDC and $CD11c^+$ $CD11b^+$ $BDCA\text{-}1^+$ DCs are analogous to CD11b cDC. These populations mainly reside in the secondary lymphoid organs and are present during steady state. Similar populations exist in peripheral tissues but they express a slightly different set of markers (7). $CD11c^+$ $CD11b^-$ $CD103^+$ DC and $CD11c^+$ $CD11b^+$ $CD103^+$ DC in the periphery are equivalent to the CD8 cDC and CD11b cDC populations respectively (13).

FUNCTIONAL DICHOTOMY IN cDC SUBSETS

The two cDC populations also have distinct functions with respect to pathogen recognition, cytokine production, and T cell activation. CD8 and CD103 cDCs express a unique pattern of TLR and other pathogen recognition receptors: they are the only subset to express TLR3 and TLR11 and also uniquely express the C-type lectin Clec9A, DEC205 (CD205), and langerin (CD207) (7, 9). CD8 and CD103 cDC are specialized in the cross-presentation of externally derived antigen on MHC class I for the activation of naïve CD8 T cells. The recent advances in the molecular mechanisms of cross-presentation are covered in a review included in this Research Topic (14). In addition, CD8 cDC appear to preferentially activate CD8 T cells due to the fact that they produce high levels of the cytokines interleukin (IL)-12 and IL-15 that are important for the differentiation and survival of CD8 T cells (15–17). CD8 and CD103 cDCs are the main producers of IL-12, an important cytokine in the differentiation of inflammatory Th1 cells (15, 16, 18). There are also reports suggesting a role for CD8 and CD103 cDCs in the maintenance of peripheral self-tolerance through the deletion of self-reactive T cells (19, 20) and the induction of Treg cells (21). While CD11b cDC are capable of cross-presentation and CD8 T cell activation they appear to be most effective in antigen presentation and activation of CD4 T cells (22) In addition the dermal and intestinal $CD11b^+$ $CD103^+$ DCs are strong inducers of Treg due to their expression of aldehyde dehydrogenase, the enzyme that converts Vitamin A to retinoic acid, necessary for Treg conversion (23, 24). Several comprehensive reviews on DC subsets and their development have recently been published and can be consulted for further details (7, 9, 13).

DC AND THE MAINTENANCE OF SELF-TOLERANCE

T and B cell tolerance to self-antigens is acquired during development through clonal deletion of self-reactive cells. DC play an important role in the process of central tolerance in the thymus as they present an array of self-antigens to developing T cells (25). If the T cells have a high affinity for self-antigen they undergo apoptosis and are deleted. Those T cells with a moderate affinity for self are induced to differentiate into forkhead transcription factor $(Foxp3)^+$ suppressor cells (26) known as thymus (t)Treg (27). In contrast, peripheral (p)Treg cells arise in the periphery upon Ag contact and several subsets of pTreg can be induced depending on the signals they encounter (28). These include IL-10 (29), TGF-β (30), retinoic acid or vitamin D3 (31, 32), low Ag dose (33), and specific DC subsets (21, 34, 35). As the study of DC biology has evolved, it has become apparent that it is possible to induce the differentiation of Treg using DCs conditioned by immunomodulatory agents (36–39). For example, the presence of TGF-β during the primary stimulation induces the production and expansion of $Foxp3^+$ Treg (35). In addition it has been possible to induce IL-10-producing T cells with DC generated using a combination of GM-CSF, TNF-α, and IL-10 (39). Studies have shown that DC producing IDO induce the generation of Treg from naïve T cells (40). Other reports have suggested that immature DC induce the development of functional suppressor cells (36) and that immature DC

might, therefore, be therapeutically useful in autoimmunity (37, 38). Recent studies using both nominal and self-Ags have demonstrated that targeting Ags to CD8 cDC in the LN or spleen in the absence of an inflammatory stimulus results in the generation of Treg (41, 42). Similar results have been obtained when Ags were targeted to $CD8^-$ $DCIR2^+$ DC (21). The CD11b cDC subset has been shown to induce Treg proliferation and expansion in the presence of GM-CSF (43). A recent paper has described a feedback loop in which the number of Tregs in the periphery can be manipulated by altering the relative number of DCs suggesting that DCs play an important role in regulating pTreg numbers (44). We have recently suggested that DC in the periphery may function to maintain tolerance by presenting endogenous self-antigens to naïve T cells thereby inducing pTreg (45).

THE ROLE OF DENDRITIC CELLS IN THE PATHOGENESIS/PREVENTION OF T1D

DC DEVELOPMENT IS DEFECTIVE IN T1D

Early studies of NOD mouse revealed abnormalities in the development of myeloid cells demonstrating a reduced proliferative capacity of the bone marrow in response to GM-CSF and M-CSF (46). Several groups, including our own, have identified defects in the development of specific DC subsets in the spleen and other lymphoid organs (47–49). These defects result in a decrease in the numbers of the CD8 cDC population (50, 51), a population which has been shown to have regulatory function (52–54), with a concomitant increase in the CD11b cDC population. Recent studies have also shown that treatment of NOD with Flt 3 ligand (FL) protects mice from diabetes development and this is correlated with an enhancement in the number of CD8 cDC (55, 56). Our own studies demonstrated that treatment of NOD mice with FL increased the number of CD8 cDC and that transfer of these cells to prediabetic NOD mice could have a partial effect in preventing diabetes (50). However a recent study suggested that the timing of FL treatment was critical since disease would be exacerbated if autoimmune CD8 T cells were already present (57).

Several studies have examined the function of bone marrow-derived and isolated DC populations with respect to cytokine production and T cell differentiation and the results have not been consistent. It has been reported by some investigators that NOD DC produce higher levels of the Th1-driving cytokine IL-12 (58–60) while others failed to find such an association (49, 50, 56). Macrophages also appear to be an important source of IL-12 in NOD mice (51, 61). Adenosine plays an important role in modulating the immune response to tissue inflammation (62, 63) and a recent study found that NOD DC express higher levels of adenosine deaminase (ADA), the enzyme responsible for catabolic degradation of adenosine, and that transfer of ADA deficient DC to NOD mice protected them from diabetes (64). These studies suggest that NOD mice have imbalances in DC subsets and alterations in DC function that may contribute to pathogenesis of T1D.

DC AND THE MAINTENANCE OF TOLERANCE IN T1D

Dendritic cells are important in both central and peripheral tolerance through the deletion of self-reactive cells and the induction of Treg. NOD mice have defects in central and peripheral tolerance mechanisms (65, 66). Recent studies have shown that the number and/or function of Treg cells decrease as NOD mice age and this is associated with onset of diabetes (67). The development and maintenance of Treg in NOD mice is highly dependent on the presence of co-stimulatory molecules CD80 and CD86 (68). In addition it was recently shown that interaction between the inhibitory molecules PD-1 on T cells and PDL-1 on DC is necessary for the maintenance of tolerance in NOD mice (69). Blockade of this interaction resulted in increased DC/T cell interaction time in the islets and T cell activation leading to the generation of autoreactive effector cells (69). Another study came to the same conclusion by using different antigen constructs to increase the time of antigen presentation (70). IL-2 and signaling via IL-2R are also critical for Treg development and maintenance (71). Recent studies have identified defects in IL-2R signaling in T1D patients (72) and the diabetes susceptibility locus, Idd3, which contains IL-2 was recently shown to control Treg function through an effect on APC (73–75). Several early reports demonstrated that the inflammatory cytokine TNF-α plays an important role in the initiation of T1D (76) and more recently this has been attributed to effects of TNF-α on DC subsets (77). In this study administration of TNF-α to NOD mice was shown to decrease the number of CD8 DC, increase the CD11b DC population, and the DC had a more mature phenotype and activated islet-specific T cells (77) A recent study describing the depletion of Treg in NOD mice showed that the increase in diabetes in these animals was associated with aggressive infiltration of pancreatic islets by DC rather than CD4 T cells (78). These results suggest that tTreg in this context may prevent autoimmunity by controlling the migration of cDC into the islet.

DC CONTRIBUTE TO THE DEVELOPMENT OF T1D

Analysis of the phenotype and function of BM-derived DC in NOD mice have suggested that these cells produce higher levels of IL-12p70, and that this related to increased expression of NFκB (58). Our own studies suggest that BM-derived DC from NOD can produce higher or lower levels of IL-12p70 depending on the culture conditions and activation stimuli used (49, 79, 80). Recent studies using DTR transgenic mice that allow the targeted depletion of macrophages or DC have demonstrated that the CD11b cDC population is responsible for presenting antigen to autoreactive T cells (81). Ablation of these cells protected NOD mice from diabetes development. The interaction of iNKT cells with cDC was shown to lead to either protection via induction of Treg or exacerbation of disease if the interaction occurred at the same time as TLR4 ligation (82).

The role of pDC in diabetes pathogenesis is somewhat more controversial and there are conflicting reports in the literature. Depletion of pDC using the CD11c-DTR system caused accelerated disease (81), suggesting a protective role for pDC. The protection induced by pDC was correlated with increased local production of IDO and increased numbers of NKT cells in the pancreas. In a model of virus infection it was shown that activation of iNKT cells stimulated TGF-β production from pDC, which led to the induction of Treg and protection from diabetes (83). In contrast, another study found that IFN-α could be detected in the pancreas of NOD mice at early ages and that blocking the ability of mice to respond to IFN-α prevented diabetes (84). Furthermore these investigators showed that depletion of pDC using a depleting

antibody reduced the incidence of diabetes (85). Another study revealed a crosstalk between B-1a B cells, neutrophils and pDC that contributed to the induction of diabetes via type 1 IFN production (86). This study suggests that DNA from dying β cells together with antibodies from B-1a B cells form complexes that are potentiated by neutrophils and lead to the induction of type 1 IFN by pDC (86). The authors show that this is seen only in NOD mice and speculate that this is related to the defects in the ability of NOD macrophages to clear debris (87). Several studies have examined the role of pDC, type 1 IFN and viral infection in the induction of T1D; reviewed in Swiecki et al. (88). The situation is complex with some studies showing exacerbation of diabetes (89, 90) with viral infection and others showing protection (91, 92). It has been suggested that whether viral infection leads to diabetes induction is related to the tropism of the virus and the local environment in which type 1 IFN is produced (88). Thus a virus infecting the pancreas may induce local type 1 IFN, which protects islets from infection and damage, whereas in other situations the type 1 IFN, produced by pDC following viral infection, could result in activation of autoreactive T cells and diabetes exacerbation (88). It has been reported that pDC also accumulate in the islets of NOD mice (93) at a later time point, around 10 weeks of age. The infiltrating pDC were shown to express IDO, which is characteristic of a more tolerogenic phenotype of DC (94) leading to the speculation that these cells may be attempting to control ongoing T cell activation. Studies in the next few years will hopefully clarify these contrasting results on the role that pDC play in diabetes pathogenesis.

A novel population of cDC, termed merocytic (mc)DC, have recently been identified and shown to be responsible for cross-presentation of islet antigens to CD8 T cells and direct presentation to CD4 T cells (95, 96). The mcDC express CD11c but are negative for both CD11b and CD8, they accumulate in the spleen as NOD mice age and were shown to secrete large amounts of type 1 IFN (95, 96). In addition transfer of mcDC pulsed with irradiated islets to non-diabetic NOD mice accelerated the onset of diabetes (95, 96). The number of mcDC was recently mapped to the *Idd13* congenic interval suggesting that the relative number of these cells is genetically determined (97). Further studies are required to determine the significance of this DC population, and whether a counterpart of these cells exists in human.

Dendritic cells are constitutively present within islets of normal mice and have been shown to express peptide-MHC (pMHC) complexes containing peptides derived from islet antigens (98). Two major DC subsets can be found within islets; the CD11b cDC and CD103 cDC (99, 100). CD103 DC depend on FL for their homeostasis whereas islet CD11b DC appear to arise from monocytes and are unaffected by the absence of FL (101). The number of DCs in the islets remains relatively stable but these numbers increase following T cell infiltration and inflammation (98, 99, 102). In addition the phenotype of the DC becomes more inflammatory with increases in the expression of co-stimulatory molecules and MHC (2). The evidence suggests that the initiation of the autoimmune response occurs within the draining pancreatic lymph node (PLN) since removal of the PLN prevents diabetes (103) and several studies have shown that the initial proliferation of islet-specific CD4 and CD8 T cells takes place in the PLN (104, 105). Islet CD103 DC migrate to the PLN where they present islet antigens to specific CD4 and CD8 T cells (2, 101), and when T cells infiltrate the islet they localize to DC-rich areas (2). The movement of DC and T cells within the islet can now be visualized using a novel two-photon imaging technique (106). In addition pancreatic CD103 DC from NOD mice have been shown to express less IL-10 than similar populations from non-diabetic strains suggesting that these have lost their ability to induce tolerance (107). Islet CD11b DC are relatively poor at presenting antigen under steady state conditions but they accumulate as inflammation increases and become more mature. Since CD11b DC do not appear to migrate to PLN their role appears to be in the modulating the local tissue response (101).

DC IN HUMAN T1D

Several studies have examined the blood of newly diagnosed T1D patients for the presence of DC subsets. pDCs have been shown to be increased (108) or decreased (109) at the time of diagnosis. A recent detailed longitudinal analysis of immune parameters in newly diagnosed T1D children has revealed that reduced numbers of cDC1s and NKT cells at the time of diagnosis are correlated with reduced residual β cell function 1 year later (110). Another study found decreased numbers of cDCs and pDCs in newly diagnosed pediatric T1D patients and also observed a decrease in the expression of CCR2 on these cells (111). Other studies have found correlations with vitamin D levels and immune cells in T1D patients (112). A study of pancreatic biopsies on a small number of new onset T1D patients revealed the presence of infiltrating macrophages and DC that produce TNF-α (113). A recent description of three cases of fulminant T1D secondary to enterovirus infection revealed marked islet infiltration of activated DC and macrophages and the presence of inflammatory cytokines (114). Thus it is likely that DCs will be shown to contribute to the onset of human T1D.

DENDRITIC CELLS AS THERAPY FOR T1D

The fact that DCs play an important role in the induction and maintenance of self-tolerance has made them attractive targets for therapeutic interventions. Three main strategies have been employed and these include the adoptive transfer of specific DC subsets, the *in vitro* expansion of Tregs with specific DCs and the *in vivo* targeting of DC subsets (115). As discussed above the complexity of DC subsets and the plasticity of their function have made this a challenging objective, since there is a fine balance between the immunostimulatory and immunoregulatory functions of DC (45, 116, 117). DCs with a so called semi-mature phenotype, which consists of increased MHC and co-stimulatory molecule expression but low inflammatory cytokine production are thought to be most effective in inducing tolerance in the context of autoimmunity (118, 119). This maturation state can be induced by exposing DC to the cytokine TNF-α and DCs treated in this way have been shown to prevent experimental allergic encephalomyelitis (EAE) (118). In general the function of these DCs is to induce the expansion of Treg or other regulatory mechanisms such as Th2 cells. The attraction for using DCs as therapeutic agents is that since they present specific antigen to T cells it is possible to envision the generation of antigen-specific tolerance (120).

ADOPTIVE TRANSFER OF THERAPEUTIC DC

Dendritic cells were first used as therapeutic agents to prevent diabetes in NOD mice over 20 years ago by Clare-Salzler et al. (121). In this study DCs isolated from the PLN of NOD mice and transferred to prediabetic NOD mice were able to prevent disease onset whereas DCs from other LN or spleen were not effective. Later studies by the same group have suggested that DC in the PLN are more mature and that this is important for their therapeutic effect (122). We have previously shown that the injection of semi-mature bone marrow-derived DC prior to the onset of destructive insulitis prevents the subsequent development of diabetes (123, 124). The therapeutic DC populations expressed high levels of co-stimulatory molecules (CD80, CD86, and CD40) and produced low levels of IL-12p70 following CD40 ligation (49). Further investigation revealed that the therapeutic DC population changed the cytokine milieu in treated NOD mice. Whereas NOD mice generally exhibit a strong bias toward type 1 cytokine production, injection of DC induced the production of type 2 cytokines (125). In addition we (124) and others (126, 127) showed that injection of DC transduced with the IL-4 gene could effectively prevent diabetes in NOD mice when given at later time points, past the onset of invasive insulitis. We also confirmed, using *in vitro* studies, that therapeutic DC could drive the differentiation of Th2 cells (79), whereas non-therapeutic DC did not. The therapeutic DCs were not treated with any tolerogenic agents and did not require the addition of islet-derived peptides but the cultures were performed in FBS, which was subsequently shown to induce a Th2 environment that may have been non-specifically contributing to the therapeutic effect (128). In more recent experiments we have shown that the therapeutic DC population induces the expansion of Treg in the presence of low dose antigen (129) and DCs cultured in the absence of FBS are equally able to induce Treg expansion (unpublished observations). In addition it has been shown that DC grown in autologous serum were able to prevent diabetes but only when pulsed with insulin-derived peptides (130).

In order to ensure that DCs maintain a tolerogenic phenotype and drive tolerance rather than immunity several approaches aimed at conditioning the DC have been explored. These have included treating DC with cytokines such as IL-10 (130, 131), IL-10/TGF-β (132), and TSLP (IL-25) (133), pharmacological agents such as dexamethasone and vitamin D_3 (134), carbon monoxide (CO) (135), anti-CTLA-4 antibody (136), secretory IgA (137) among others. In general, the treatment of DCs with these agents induces a semi-mature phenotype in the DC characterized by reduced expression of co-stimulatory molecules, reduced inflammatory cytokine production and resistance to further maturation stimuli. The infusion of these modified DC into NOD mice results in the deletion of autoreactive CD4 T cells and the generation of islet-specific Treg (130, 131, 133, 136, 137). One exception to this was the treatment of NOD mice with CO-treated DC, which reduced β1-integrin expression by CD8 T cells and thus inhibited their migration into the islet (135). Another approach has been the use of anti-sense oligonucleotides to down-regulate the expression of the co-stimulatory molecules, CD40, CD80, and CD86 on DC (138). This approach prevented diabetes in NOD mice through the induction of Treg and a phase 1 clinical trial using this approach was initiated (139).

DC USED TO EXPAND REGULATORY T CELLS *IN VITRO*

Dendritic cells have also been used *in vitro* in order to expand Treg cells, and these Treg can be adoptively transferred into individuals with autoimmunity with the hope of curbing or arresting the inflammatory response. An early study expanded Treg, isolated from an islet-specific TCR transgenic NOD mice (BDC2.5), with peptide pulsed DC and IL-2 and showed that these cells could prevent diabetes in prediabetic NOD mice (34). Further studies from this group indicated that DC expressing higher levels of the co-stimulatory molecule, CD86 were the most efficient at inducing Treg expansion (140). The CD11b cDC subset has been shown to induce Treg proliferation and expansion in the presence of GM-CSF (43). A recent study examining the mechanism by which GM-CSF treatment prevents diabetes has further suggested that the expression of OX40L and the Notch3 ligand Jagged1 on DC was necessary for Treg expansion *in vitro*, although this study did not determine whether these polyclonal expanded Treg were effective in preventing diabetes (141). Some of the approaches to generate tolerogenic DC are being developed for clinical trial in the context of transplantation (142) but these are not yet being fully developed for use in autoimmune disease. A recent study compared the ability of several DC conditioning regimens to induce suppressive Treg in the human system (143). This study found that DC pretreated with IL-10 induced the most suppressive Treg, while TGF-β, rapamycin, and dexamethasone were less effective (143).

IN VIVO TARGETING OF DC

In general these types of therapies have involved the use of reagents that deliberately target specific DC subsets but these can also include approaches that, while not directly targeting DC, act by changing the local DC environment. Recent studies using both nominal and self-antigens have demonstrated that targeting antigens to CD8 cDC in the LN or spleen using anti-CD205 antibodies coupled to specific antigens in the absence of an inflammatory stimulus results in the generation of Treg (41, 42). Similar results have been obtained when antigens were targeted to CD11b cDC (21). A recent study used anti-CD205 antibodies coupled to the peptide mimetope for the CD4 BDC2.5 T cell to target DC in NOD mice (144). This resulted in the generation of long-lived and stable BDC2.5 Treg but this had no impact on the development of diabetes. However when the anti-CD205 was coupled to the proinsulin protein diabetes was prevented (144). Similar studies using an anti-CD205 coupled to HA and given to INS-HA/TCR-HA mice also demonstrated efficacy in the prevention of diabetes (145). In this mouse model the foreign antigen HA is expressed in the pancreas under the control of the rat insulin promoter and the T cells are all transgenic for an HA-specific TCR (145). These results suggest that the choice of antigen for targeting will be critical if this approach is to move forward. A similar approach has been used to induce tolerance in islet-specific CD8 T cells. These studies reported initial activation of adoptively transferred islet-specific CD8 cells followed by deletion of these cells, but the impact of these on diabetes development was not reported (146).

It has been known for some time that varying the dose of the stimulating antigen has a profound effect on T cell differentiation (147–149). Interest in this area has been renewed by the observation that the induction of Treg is optimal in situations of low

TCR signal strength (33, 129, 150). The induction of Treg by low dose antigen occurs optimally when DC are presenting the antigen (129), and while the addition of TGF-β is not necessary a recent study showed that TGF-β production by T cells is required (151). Interestingly the most effective induction of Treg *in vivo* occurs when low doses of a high affinity peptide are used, rather than higher doses of low affinity peptides (152). Furthermore it was recently shown that the affinity of the peptide affects the duration of DC/T cell contacts *in vivo* providing an explanation for why low dose of more potent pMHC complexes are most effective at driving Treg expansion *in vivo* (153). Several approaches have been attempted to deliver low dose antigen *in vivo* including the continuous delivery of low dose antigen via osmotic pump (154) and the direct targeting of DC subsets as discussed above (21, 41, 42). In the context of T1D a recent study demonstrated that low doses of a high affinity insulin peptide mimetope was more effective at preventing diabetes than the native peptide (155). These results suggest that long-lasting peripheral tolerance could be achieved by delivering the appropriate dose of antigen to steady state DC in the periphery. The challenge will be translating this to the human situation although a recent paper showed that human insulin-specific Treg could be induced by low dose stimulation (156).

Another way to target DC *in vivo* is to use microparticles (157). A recent study used microspheres loaded with anti-sense oligonucleotides specific for CD40, CD80, and CD86 and showed prevention of diabetes along with an increase in Treg in treated NOD mice (158). These investigators further showed that repeated treatment with these microspheres could reverse diabetes in newly diagnosed NOD mice. The microspheres were taken up by resident DC in the spleen and these DC showed reduced expression of the co-stimulatory molecules (158).

In a study of combined therapy with oral anti-CD3 and intravenous anti-CD20 antibodies the prevention of diabetes seen in these animals was correlated with an increase in Treg and IL-10-producing T cells. Furthermore a population of IL-27 producing DCs was found to be responsible for the induction of IL-10-producing T cells in this system (159). As discussed above FL treatment of NOD mice in some circumstances prevents T1D (55, 56) but the timing of FL administration has to be before the onset of autoimmunity otherwise disease is exacerbated (57). Treatment of NOD mice with soluble CTLA-4 has been shown to restore the tolerance inducing properties of DC through the IFN-γ dependent activation of IDO. In this model CTLA-A binds to B7 molecules on DC and this stimulates the release of IFN-γ, which activates the immunosuppressive mechanism of tryptophan catabolism (160). A novel approach has been to induce accumulation of thymic DC populations which results in an increase in suppressive Tregs. A recent study identified the delta-like ligand 4 (Dll4)-Notch signaling pathway as important in controlling the number of thymic DCs (161). These investigators found that blockade of the Dll4-Notch pathway resulted in an increase in thymic DC numbers and thymic Treg. Treatment of NOD mice with these blockers not only prevented the establishment of disease in prediabetic animals but also was able to reverse disease in newly diagnosed diabetic animals (161). This protection was dependent on the presence of Treg since treatment of non-diabetic animals with a Treg-depleting antibody reversed the protection (161). Several studies have reported the use of α-GalCer, an agonist for iNKT cells, as a means to prevent disease in NOD mice (162–164). Recent studies have suggested that α-GalCer treatment induces tolerogenic DC populations that induce non-inflammatory islet-specific T cells (165), and novel derivatives of α-GalCer have been developed that have the same effect without some of the profound suppressive effects on iNKT function (166).

CLINICAL TRIALS IN T1D INVOLVING DC

As discussed above a phase I clinical trial utilizing autologous monocyte-derived DC treated with anti-sense oligonucleotides to down-modulate co-stimulatory molecules has been conducted. In this trial of 10 patients with established T1D, 7 were given the immunosuppressive DC and 3 were given unmanipulated DC. In this trial no adverse events were observed and there were few changes noted in terms of insulin requirements, immune cell phenotype, and cytokine production (139). Interestingly, further analysis of this clinical trial revealed an increase in certain B cell populations and subsequent *in vitro* studies revealed that human DC treated with these anti-sense oligonucleotides could induce the proliferation of immunosuppressive B cells (167). A second trial using this same approach is now ongoing but results are not yet available. The field of DC-based vaccines is much more advanced in the setting of cancer and many clinical trials have been conducted; reviewed in the current Research Topic (Butterfileld, under review). Many features of cancer DC vaccines have been studied and it has become apparent that characteristics such as the form of the antigen used to pulse the DC, the conditioning regimen of the DC, and the route of administration all play critical roles in determining the outcome of the DC-based immunization. These features will need to be taken into consideration as more DC-based therapies are proposed for the treatment of T1D. There have been numerous clinical trials in T1D with the aim of inducing antigen-specific tolerance (168). These trials utilize islet-derived antigens, such as insulin GAD65 or hsp 70, and various routes of administration have been tested including oral, nasal, and intradermal. So far these trials have failed to have a significant impact on disease although in some cases evidence for immune tolerance was observed (168). It is to be expected that the success of such trials will depend on the APC, most likely a DC, targeted by these antigen formulations. At the present time not much attention is being paid to the nature of the DC presenting the antigen in these trials but, in view of our increasing understanding of the complexity of DC phenotype and function this is likely to change.

FUTURE PERSPECTIVES

As discussed at the beginning of this review many of the studies that examine the therapeutic potential of DC in the treatment/prevention of T1D have been performed in the NOD mouse. This has been a very useful model for identifying susceptibility genes and understanding the progression to disease but therapeutic approaches that have successful in this mouse model have not translated well to the clinic (169). DCs play a pivotal role in setting the tone of the immune response. On the one hand they contribute to the development and maintenance of self-tolerance and on the other they contribute to the breaking of that tolerance and the initiation of disease. A deeper understanding of how DC

can influence the development of autoimmune diabetes will aid in the development of novel therapeutic strategies. A great deal of progress has been achieved in the last several years and we have a better understanding of how DC can both maintain and break self-tolerance. The challenge in the future will be to use this knowledge to achieve the ultimate goal of inducing antigen-specific tolerance to prevent autoimmunity without causing widespread immune suppression.

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Regulatory dendritic cells for immunotherapy in immunologic diseases

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We recognize well the abilities of dendritic cells to activate effector T cell (Teff cell) responses to an array of antigens and think of these cells in this context as pre-eminent antigen-presenting cells, but dendritic cells are also critical to the induction of immunologic tolerance. Herein, we review our knowledge on the different kinds of tolerogenic or regulatory dendritic cells that are present or can be induced in experimental settings and humans, how they operate, and the diseases in which they are effective, from allergic to autoimmune diseases and transplant tolerance. The primary conclusions that arise from these cumulative studies clearly indicate that the agent(s) used to induce the tolerogenic phenotype and the status of the dendritic cell at the time of induction influence not only the phenotype of the dendritic cell, but also that of the regulatory T cell responses that they in turn mobilize. For example, while many, if not most, types of induced regulatory dendritic cells lead $CD4^+$ naïve or Teff cells to adopt a $CD25^+Foxp3^+$ Treg phenotype, exposure of Langerhans cells or dermal dendritic cells to vitamin D leads in one case to the downstream induction of $CD25^+Foxp3^+$ regulatory T cell responses, while in the other to $Foxp3^-$ type 1 regulatory T cells (Tr1) responses. Similarly, exposure of human immature versus semi-mature dendritic cells to IL-10 leads to distinct regulatory T cell outcomes. Thus, it should be possible to shape our dendritic cell immunotherapy approaches for selective induction of different types of T cell tolerance or to simultaneously induce multiple types of regulatory T cell responses. This may prove to be an important option as we target diseases in different anatomic compartments or with divergent pathologies in the clinic. Finally, we provide an overview of the use and potential use of these cells clinically, highlighting their potential as tools in an array of settings.

Keywords: dendritic cell, tolerance, regulatory T cell, immunoregulation, IL-10, retinoic acid, TGFβ, vitamin D

During the 1960s, it was thought that macrophages, with their capacity to phagocytose antigens, were required to initiate immunity to foreign substances (1). It was known that lymphocytes were mediators of immunity, but we knew little about how antigens from an invading pathogen would reach the lymph node-sequestered naïve lymphocytes (2). There was a gap in the understanding of the initiation of adaptive immunity, a gap that Ralph Steinman and Zanvil Cohn set out to fill. When Steinman began to study the spleen and lymph nodes, he observed new cells that were distinct from macrophages in appearance and function. These dendritic cells, so named because of their dendrite-like projections, had few lysosomes and only moderate phagocytic activity (3, 4), but they expressed high levels of major histocompatibility complex (MHC) molecules required for presentation of extra-cellular antigens (5). He also observed that dendritic cells were highly potent immune stimulators (6), and now we often speak of dendritic cells as the most proficient of professional antigen-presenting cells (APCs). By 1991, we had accumulated substantial knowledge on the role of the dendritic cell in the induction of immunity, but we were just beginning to recognize that extrathymic dendritic cells could also play central roles in the induction of tolerance (7), and it was not long before we began to understand more about tolerogenic dendritic cells and their potential applications (8–10). We now appreciate that there are numerous discreet populations of naturally occurring regulatory dendritic cells, but focusing on understanding the immunobiology of these cells within their individual niches has given us substantial insights on how we can generate and employ regulatory dendritic cells for immunotherapeutic applications. While dendritic cells can activate either $CD4^+$ or $CD8^+$, or even $CD4^-CD8^-$ T, B, and NK cells to become regulatory cells, this review will be confined to a discussion on tolerogenic DC in the context of $CD4^+$ T cells and their responses. We will first describe

Abbreviations: AHR, airway hyperresponsiveness; APCs, antigen-presenting cells; BAL, bronchoalveolar lavage; BDCA, blood dendritic cell antigen; CTLA4, cytotoxic T lymphocyte antigen-4; DC10 or DC-10, semi-mature or immature, respectively, IL-10-differentiated dendritic cells; DC-SIGN, dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin; Foxp3, the transcription factor forkhead box P3; ICOS and ICOS-L, inducible costimulator and inducible costimulator ligand, respectively; IDO, indoleamine-2,3-dioxygenase; ILT, immunoglobulin-like transcript; iTreg, induced $CD25^+Foxp3^+$ regulatory T cells; MHC, major histocompatibility complex; nTreg, naturally-occurring $CD25^+Foxp3^+$ regulatory T cells; OVA, ovalbumin; RALDH2, retinaldehyde dehydrogenase 2; Teff cell, effector T cell; TLR, toll-like receptor; Treg cell, regulatory T cell.

the populations of dendritic cells found *in vivo* and then look at the major populations of regulatory dendritic cells that have been induced *ex vivo*, as well as the effector molecules employed by these cells.

OVERVIEW OF DENDRITIC CELL BIOLOGY

In general, dendritic cells express MHCII but lack T cell (CD3), B cell (CD19), and NK cell (CD56) lineage markers (11); some subsets of dendritic cells express the monocyte/macrophage (CD14) or NK cell/neutrophil and monocyte/macrophage (CD16) lineage markers, and others the CD4 and/or CD8 T cell subset markers. Dendritic cells are formed from bone marrow progenitors that in general give rise to circulating dendritic cell precursors (12, 13) that seed the peripheral tissues as immature cells (14). As quiescent or immature cells, they express receptors for, and have an innate capacity to respond to an array of inflammatory signals, including ligands for toll-like receptor (TLR), NOD-like receptors, and scavenger receptors, as well as inflammatory mediators, cytokines, and chemokines. The various sub-populations of dendritic cells can respond in a qualitatively and quantitatively distinct fashion to such environmental triggers and differentiate extensively to become immunocompetent accessory cells, such that they provide a crucial link between the innate and adaptive immune responses (15). They upregulate cell-surface expression of their antigen-presentation machinery, including processed antigen peptide-loaded MHCII (16) and co-stimulatory molecules as well as receptors for lymph node-homing chemokines (e.g., CCR7), and they downregulate their phagocytic activities and receptors for local inflammatory signals (e.g., CCR5, CCR6) (14, 17). As dendritic cells mature, they lose their ability to process new peptides (18, 19) and migrate to their tissue-draining lymphoid organ, where they present their processed antigens to T cells in the context of cell-surface MHC (APC signal 1) together with supporting co-stimulatory molecules (e.g., CD40, CD86; APC signal 2) and T cell-polarizing cytokine signals such as IL-12 (20) (APC signal 3), inducing the T cells to differentiate into antigen-specific effector T cells (Teffs; e.g., Th1, Th2, or Th17 cells) (13). But dendritic cells can also provide a fourth APC signal of sorts to T cells, by which they direct the trafficking of the educated T cell. In the gut, retinoic acid and transforming growth factor (TGF)-β produced by dendritic cells together induce T cells to express the α4β7 and CCR9 gut-homing receptors (21), while in the skin-draining lymph nodes vitamin D metabolites released by the dendritic cell induce T cells to express CCR10, such that they become responsive to the skin-homing chemokine CCL27 (22).

Tissue-resident dendritic cells that acquire innocuous environmental or self antigens in the absence of local inflammatory responses similarly migrate to the draining lymph nodes but, as more quiescent cells, overall they express lower levels of MHCII, co-stimulatory molecules, and IL-12, and secrete instructional regulatory mediators such as IL-10 or retinoic acid (23, 24). In this way dendritic cells that are presenting innocuous environmental antigens activate one of several types of regulatory T cell (e.g., Treg, Tr1, or Th3) responses that are associated with immune tolerance (**Figure 1**).

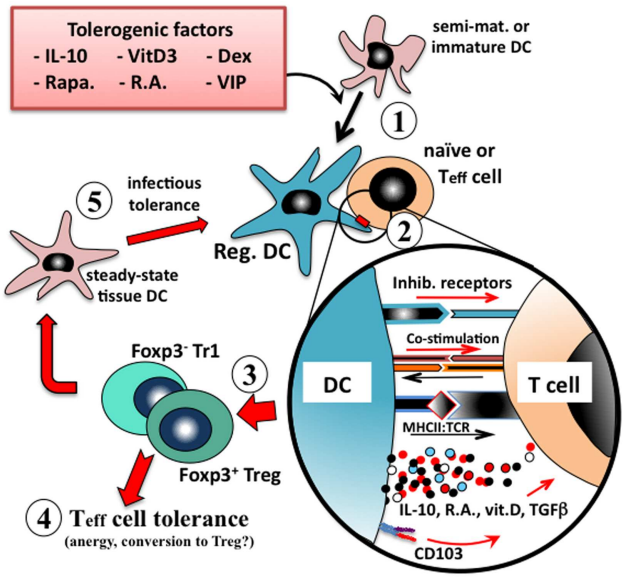

FIGURE 1 | Induction of immunologic tolerance by regulatory dendritic cells. Immature or semi-mature dendritic cells that are incubated with, or differentiated in the presence of, tolerogenic factors (e.g., IL-10, vitamin D3, corticosteroids, or retinoic acid) (1) adopt a regulatory phenotype. When these converted regulatory dendritic cells are pulsed with antigen and exposed to cognate naïve or effector T (Teff) cells (2), they present their processed antigen peptides in the context of MHCII, and also lower levels of co-stimulation (e.g., CD40, CD86) to the T cells, but at the same time many types of tolerogenic cells also provide inhibitory receptor (e.g., ILT2, ILT4) signaling to the T cell. Counter-signaling from the engaged T cell activates dendritic cell production of polarizing mediators (e.g., IL-10, TGFβ), which together instruct the T cell to adopt a regulatory phenotype. The nature of the instructional signals from the dendritic cell to the T cell determine whether it adopts an IL-10-secreting $CD25^{+}Foxp3^{+}$ Treg phenotype or an IL-10/TGFβ-secreting $Foxp3^{-}$ Tr1 phenotype (3). These regulatory T cells are able to suppress the responses of cognate or by-stander naïve or effector T cells in their microenvironment (4) and also to convert endogenous tissue dendritic cells to adopt a regulatory phenotype through induction of infectious tolerance (5), and thereby reinforce the tolerance phenotype.

NATURALLY OCCURRING POPULATIONS OF DENDRITIC CELLS

A large number of reports have described an array of dendritic cell types and subtypes in different organ systems and animals, and it is almost undoubtedly true that more will be described as we explore further. Many of these sub-populations are or can be tolerogenic as they are found in their steady state (e.g., pulmonary plasmacytoid or myeloid dendritic cells), but for most if not all of these there are inflammatory signals that can override this tolerogenic phenotype, converting these cells to an immunostimulatory phenotype. In some tissues (e.g., gut, liver) dominantly tolerogenic signals are constitutively expressed at high levels, while in other sites that are not routinely exposed to the external environment these signals may be much more subtle.

DENDRITIC CELLS IN THE BLOOD

Several distinct types of dendritic cells can be identified in human peripheral blood. There are two sub-populations

of MHCII$^+$CD11c$^+$CD123^{lo} myeloid dendritic cells, including CD1c/blood dendritic cell antigen (BDCA)-1$^+$ cells and CD141/BDCA-3$^+$ cells, as well as MHCII$^+$CD1c$^-$CD123^{hi} plasmacytoid dendritic cells that also express BDCA-2/CD303, BDCA-4/CD304, IL-3RA, and ILT7 (11, 25). The CD141$^+$MHCII$^+$CD11c$^+$ myeloid dendritic cell is the human counterpart of the murine CD8α$^+$ dendritic cell (25). In the mouse, the identities of circulating tissue dendritic cell precursor(s) have not been all that well documented (26). We know that murine splenic and lymph node dendritic cells are continuously replaced from a pool of blood-borne precursors (27), that splenic CD8α$^+$ dendritic cells most likely gain access to this organ via the vasculature (28), and that MHCII^{lo}CD11c^{lo} pDC do accumulate in the blood of mice (13, 28). While immunostimulatory (29) and tolerogenic (30) dendritic cells can be readily differentiated *ex vivo* from peripheral blood monocytes in humans, it was only recently that LPS stimulation of murine monocytes was reported to induce dendritic cell differentiation (31). These murine monocyte-derived dendritic cells express CCR7 and dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and localize to T cell areas of lymph nodes, where they are highly effective in presenting and cross-presenting antigens (31).

In humans, the BDCA-1$^+$ and -3$^+$ myeloid dendritic cell populations can be mobilized from the bone marrow with Flt3 ligand alone while optimal plasmacytoid dendritic cells mobilization reportedly calls for use of Flt3 ligand and G-CSF (25). The circulating BDCA-1$^+$/CD1c$^+$ myeloid dendritic cell can secrete abundant IL-12 and prime cytotoxic T cell responses (32), while BDCA-3$^+$ myeloid dendritic cells and BDCA-2$^+$ plasmacytoid dendritic cells instead secrete IFNγ and IFNα, respectively, on activation (32). A minor population of tolerogenic IL-10-expressing CD1c$^-$CD303$^-$CD14$^+$ dendritic cells has recently been described in human peripheral blood, although much of the data regarding their tolerogenic activities has come from studies with an *in vitro* analog of the circulating cell (33).

INTESTINAL DENDRITIC CELLS

The intestinal immune system routinely faces the challenge of discriminating pathogens from harmless commensal organisms and other (e.g., food) antigens, as a prelude to triggering effector and regulatory T cell responses, respectively (34). The gut-associated dendritic cells include those in the mesenteric lymph nodes (MLNs), intestinal lamina propria, and the isolated lymphoid follicles (35, 36). The lamina propria contains two populations of CD11c$^+$ mononuclear cells, including CD11c^{hi}CD103$^+$CD11b$^+$CX$_3$CR1$^-$ cells and CD11c^{int}CD103$^-$CD11b$^+$CX$_3$CR1$^+$ cells; the CD103$^+$ cells are *bona fide* dendritic cells while the latter CD103$^-$ cells are now thought to be resident tissue macrophages (37). Under steady-state conditions, the CD103$^+$ dendritic cells express retinaldehyde dehydrogenase 2 (RALDH2) (23, 38), TGF-β (39), and indoleamine-2,3-dioxygenase (IDO) (40), such that targeting of antigens to these cells leads to tolerance outcomes, while gut inflammation dampens TGFβ and RALDH2 expression in these cells, such that they instead induce vigorous T and B cell responses (41, 42). CD103, the α chain of the E-cadherin ligand αEβ7 integrin (43), is expressed on almost all lamina propria dendritic cells and a subset of MLN dendritic cells (44). It has been reported that gut luminal bacteria recruit lamina propria CD103$^+$ dendritic cells into the gut epithelium, from which they extend filipodia into the lumen to sample gut antigens (37). RALDH2 is an enzyme that catalyzes the synthesis of retinoic acid, a vitamin A derivative, which plays a major role in immunologic tolerance within the gastrointestinal tract (45). Expression of CD103 and retinoic acid together induce gut T cells to express the gut-homing receptors CCR9 and α4β7 (44, 46). CCR9 and its CCL25 ligand regulate recruitment of lymphocytes to the vasculature of the small intestine (47), while α4β7 integrin expression confines extravasation of these T cells to the intestinal post-capillary venules (48). Retinoic acid and TGFβ together promote the differentiation of Foxp3$^+$ Treg from naive T cells (39), while retinoic acid further reinforces tolerance by dampening Th17 cell differentiation (49). Retinoic acid also fosters B cell isotype switching to IgA antibodies as well as their expression of CCR9 and α4β7 (50–52), and thereby contributes further to local tolerance responses.

PULMONARY DENDRITIC CELLS

Pulmonary dendritic cells can be differentially positioned in either the conducting airway or the interstitium of the lung (15, 53). In mice, CD11c^{hi} myeloid cells are found in both compartments, while CD11c$^-$ cells are reportedly confined to the airway mucosa (53). The airway dendritic cells form a prototypical network of interdigitating cells positioned beneath the epithelium (54–56), with many of these cells extending dendritic processes into the airway lumen to sample airway antigens (57), just as occurs in the gut (37). In mice these airway cells express CD11c$^+$, MHCII$^+$, and CD11b$^+$, but not CD8α$^-$ (15); they also express CD103 and tight junction proteins (claudin-1 and -7, and zonula occludens protein 2), which would play important roles vis-à-vis their positioning within the epithelium (43). After airway antigen sampling and processing, these cells can activate cognate T cells in their immediate environment (57, 58), but also migrate to the lung-draining lymph nodes where they present to T cells in that compartment (58). In rats the airway-associated dendritic cells are somewhat more heterogeneous (53). Bronchoalveolar lavage (59) and tissue digest (60) studies of the human lung have revealed three populations of dendritic cells, including CD11c$^+$CD1c$^+$ and CD11c$^+$BDCA-3$^+$ myeloid cells, and CD11c$^-$BDCA-2$^+$ plasmacytoid dendritic cells, and these are considered analogous to the CD11b$^+$CD103$^-$ and CD11b$^-$CD103$^+$langerin$^+$ conventional and plasmacytoid dendritic cell subsets, respectively, in mice (61). Further analysis in chronically inflamed (e.g., COPD) lung tissues have revealed langerin-positive and DC-SIGN-expressing dendritic cell sub-populations (31, 62) that were proposed to represent the human equivalent of the murine CD11b$^-$CD103$^+$langerin$^+$ and monocyte-derived inflammatory dendritic cells, respectively (31, 62). The CD103$^+$ dendritic cells that comprise the bulk of the dendritic cells found in the lung-draining lymph node migrate there from the lung mucosa under the influence of lymph node-homing chemokines that signal via the CCR7 (43). In humans, the lung plasmacytoid dendritic cells express CD123 and BDCA-2, while the mouse plasmacytoid dendritic cell is B220^{hi}Ly6C^{hi}Gr1^{lo}CD11b$^-$CD11c^{lo} (63). Plasmacytoid dendritic cells, which contribute importantly to tolerance responses to

innocuous airway antigens (64), also express CD45RA, Ly49Q, BST2/tetherin [or murine plasmacytoid dendritic cell antigen (mPDCA)], sialic acid-binding immunoglobulin-type lectin (siglec)-H, inducible costimulator ligand (ICOS-L), programed death 1 ligand (PD-L)-1, and IDO (65), but produce copious amounts of IFNα in response to viral challenge (66). Under tolerogenic conditions, the $CD103^-$ and $CD103^+$ dendritic cells reportedly are specialized in presenting antigen to $CD4^+$ versus $CD8^+$ T cells, respectively (67, 68). However, under viral challenge the $CD103^+$ dendritic cells efficiently migrate to the draining lymph nodes where they cross-present viral antigens to $CD8^+$ T cells, while the $CD103^-$ cells tend to remain within the lung parenchyma, where they present to $CD4^+$ T cells in a pro-inflammatory manner (67, 68). This separation of function is also observed in asthmatic animals, wherein the $CD103^-$ dendritic cells present allergen to parenchymal $CD4^+$ T cells, while the $CD103^+$ subset presents allergen in the draining lymph node (69).

It is clear that the pulmonary dendritic cell contributes not only to the induction of asthma, but also to allergen-tolerance. Wholesale depletion of $CD11c^+$ cells abolishes disease onset following allergen exposure in experimental animals (70), but plasmacytoid dendritic cell depletion in animals challenged with otherwise innocuous aeroallergens leads to development of allergen-specific asthmatic responses (64). Steady-state plasmacytoid dendritic cells express an immature/semi-mature phenotype, with low levels of MHCII and co-stimulatory molecules and intermediate levels of PDL-1 (15, 71), which would contribute to their tolerogenic phenotype, but IDO expression by these cells also strongly inhibits T cell proliferative responses (72). Nevertheless, $CD103^+$ dendritic cells from the lungs of allergen-tolerant mice would also affect tolerance, inasmuch as they express RALDH and secrete retinoic acid, which contributes together with TGFβ to local induction of $Foxp3^+$ regulatory T cells (73). Finally, it is important to note the contributions of other populations within the lung to tolerance. Tissue-resident (74) and alveolar (75) macrophages both express TGFβ and RALDH under steady-state conditions, such that they can also induce $CD4^+$ T cells to which they present innocuous antigens to convert into $Foxp3^+$ Treg. Alveolar macrophages can also suppress the immunostimulatory properties of steady-state lung-resident dendritic cells (76) and thereby further contribute to steady-state tolerance in the lung.

CUTANEOUS DENDRITIC CELLS

As with the intestinal tract and lungs, the skin is constantly exposed both to pathogens, which require induction of protective Teff responses, and to innocuous environmental agents for which tolerance is the desired outcome. There are at least three subsets of skin-derived dendritic cells, including the self-renewing epidermal $langerin^+CD103^-$ Langerhans cell (77), and the $langerin^+CD103^+$ (78, 79) and $langerin^-CD103^-$ (80) dermal subsets; others have reported that the dermis contains five distinct subsets of dendritic cells (81). The epidermal Langerhans cell is probably the best known dendritic cell – as in other interfaces with our environment, these superficial cells form a contiguous network of interdigitating cells that are well positioned to detect and respond to cutaneous insults (82). In general, skin dendritic cells that acquire local antigens for lymph node presentation downregulate their E-cadherin epithelial receptors and upregulate CCR7, thereby acquiring responsiveness to chemokines expressed in the T cell zones of the draining lymph nodes (e.g., CCL19, CCL21) (14). In the lymph node, the dendritic cell presents its processed antigen peptides to the T cell, along with its co-stimulatory and polarizing signals. In addition, vitamin D3 metabolites expressed by the antigen-presenting dendritic cell induces T cell upregulation of CCR10, the receptor for the skin-homing chemokine CCL27 (22).

$Langerin^+$ migratory skin dendritic cells (i.e., $CD103^+$ dermal dendritic cells and Langerhans cells) can promote T cell tolerance responses to self antigens (83). The Langerhans cell appears to be unique in some respects, however, such that exposure to potent inflammatory adjuvants by itself does not override their innate tolerogenicity (84), perhaps in part because they do not express a number of important microbial pattern recognition receptors (e.g., TLR2, TLR4, or TLR5) (85). They are also unique in that, even while in a tolerogenic mode, they strongly express APC co-stimulatory markers and express IL-12. Nevertheless they fail to effectively activate NF-κB (i.e., translocate RelB into the nucleus) following adjuvant exposure (84), which is critical to induction of the immunostimulatory phenotype in dendritic cells (86). While dermal dendritic cells can effectively induce anti-bacterial immune responses, and would need to do so in situations where microbial organisms successfully penetrate the epithelial barrier, the default function of the Langerhans cell instead leads to regulatory T cell responses, perhaps as a means of preventing the integrity of the epidermal barrier from being compromised (85). Indeed, Langerhans cell depletion (e.g., by UV-B light exposure) has long been recognized to augment pathology in multiple contact sensitivity settings (87, 88). The Langerhans cell is efficient at capture and presentation of contact irritants, but this process can culminate in anergy and/or deletion of responding $CD8^+$ T cells, with induction of $ICOS^+CD4^+Foxp3^+$ regulatory T cell responses (89). The resident $CD141^+$ dermal dendritic cell in humans can also effect tolerance through their expression of the inhibitory receptor ILT3 and of IL-10, which together upregulate $CD25^+$ regulatory T cells that protect against allograft rejection (90). Migratory $CD103^+langerin^+$ dermal dendritic cells can also induce $CD25^+Foxp3^+$ Treg outgrowth from naïve T cells, at least in part through their expression of TGFβ (91).

HEPATIC DENDRITIC AND OTHER TOLERANCE-PROMOTING CELLS

It is well recognized that operational tolerance occurs more frequently with liver transplants than with other organs, suggesting that this organ may have a unique tolerogenic capacity (92). Human liver dendritic cells comprise most prevalently $BDCA\text{-}1^+$ DC that, unlike blood dendritic cells, secrete substantial amounts of IL-10 on TLR ligation, and this contributes to their high level induction of $CD25^+Foxp3^+$ Treg (11). It has also been reported that, in the steady state, hepatic myeloid and plasmacytoid dendritic cells can both induce tolerogenic T cell responses, although by distinct mechanisms – the myeloid cells express a mature phenotype and produce high levels of regulatory factors such as IL-10, IL-27, retinoic acid, and prostaglandin E2 (93–96), whereas hepatic plasmacytoid dendritic cells express a more immature phenotype and secrete high levels of IL-10 (97, 98). The non-parenchymal

hepatic stellate cell, the major storage site for retinol in the body (99), would potentially also play a role in hepatic tolerance through provision of retinoic acid and thereby by-stander contributions to hepatic regulatory T cell induction (100, 101). Another factor to consider in hepatic tolerance is the resident liver macrophage, the Kuppfer cell. Kuppfer cells are present in very large numbers in the liver and express MHCII and co-stimulatory molecules, although as quiescent cells they only poorly present antigen. Nevertheless, just as the hepatic stellate cells are a rich source of retinoic acid, Kuppfer cells constitutively express abundant prostaglandin E2 and 15d-prostaglandin J2, which strongly inhibit T cell responses to immunostimulatory dendritic cells (102). Thus, there are multiple mechanisms that may contribute to the innate tolerogenic phenotype of the liver.

EXPERIMENTAL APPLICATION OF TOLEROGENIC DENDRITIC CELLS

STEADY-STATE AND IMMATURE DENDRITIC CELLS

For practical reasons it is unlikely that steady-state dendritic cells freshly purified from donor tissues would be used clinically, but investigations into such cells have provided substantial insights into the immunobiology of tolerogenic dendritic cells. Steady-state dendritic cells from lymphoid organs (103, 104) and non-inflamed tissues (91, 104, 105) express a relatively immature phenotype – in general, such cells are tolerogenic (105, 106). For example, treatment with small numbers of antigen-pulsed steady-state CD8α$^+$ splenic dendritic cells can induce asthma tolerance in mouse models, reversing the asthmatic animals' bronchial hyperresponsiveness and airway eosinophil and Th2 cytokine recall responses to allergen challenge; expression of IL-10, TGFβ, and IDO, as well as direct dendritic cell–Teff cell contact each contribute to the tolerogenic activities of these cells (107). It is important to their activity that such steady-state dendritic cells remain quiescent while being purified or manipulated *ex vivo*, as even overnight exposure of CD8α$^+$ dendritic cells to GM-CSF, for example, converts them into potent inducers of cytotoxic CD8$^+$ T cell responses (108). Steady-state CD8α$^+$ dendritic cell signaling leads to attenuated IL-2 expression by T cells and increased apoptosis, at least in part through the dendritic cell's expression of FasL (109–111).

Tissue dendritic cells that acquire antigens *in situ* in such a way that they do not become activated also remain tolerogenic. Thus, as noted, steady-state airway mucosal dendritic cells routinely migrate to the draining lymph nodes and present innocuous allergens in a tolerogenic fashion – indeed, this is the default mechanism by which ≈80% of the human population remains allergen-tolerant (112). Similarly, targeting antigens to dendritic cells with anti-DEC205, for example, does not activate the cells and thus leads to antigen-specific tolerance in multiple models (113–115). And dendritic cells that phagocytose apoptotic cells remain in a largely quiescent state and thus are also tolerogenic (116, 117), at least in part via induction of TGFβ expression in the draining lymph nodes with consequent activation of Foxp3$^+$ Treg (118).

There is also a large body of data regarding the tolerogenic properties of immunologically immature dendritic cells that have been generated *in vitro* from bone marrow or blood of mice or humans. These cells tend to express low levels of MHCII and co-stimulatory markers and have thus been thought of as largely ineffective in activating T cells through the classical TCR signaling pathways (119–122), although it has also been suggested that PD-L1 and PD-L2 expression by these cells contributes to their tolerogenic activities (123). There are ≈100 genes that are differentially expressed in immature versus immunostimulatory mouse bone marrow-derived dendritic cells, including a number of cytokines (e.g., Flt3L, TNF), chemokines (e.g., MIP2, RANTES), chemokine receptors (e.g., CCR2, CCR5), and other (e.g., RP105, Ax1) markers (124). Passive transfer of antigen-pulsed immature dendritic cells has been shown to induce tolerance either *in vivo* or *in vitro* in numerous experimental models and with human cells (125–132). An important caveat with use of immature dendritic cells to treat overtly inflammatory conditions is that the pro-inflammatory environment they face *in vivo* can activate these cells, such that they activate pathogenic (e.g., Th1, Th17) as opposed to regulatory T cell responses (133, 134), as discussed below.

INDUCED TOLEROGENIC DENDRITIC CELLS

Some of the first insights into the induction of a tolerogenic phenotype within dendritic cells arose from the studies of Langerhans cells that had been exposed to either ultraviolet B radiation (8) or IL-10 (8, 9). Dendritic cells from IL-10-expressing melanoma tumors (135) and IL-10-exposed immature monocyte-derived dendritic cells (136) were then also shown to be tolerogenic. This potential for using tolerogenic cells, whether dendritic cells or subsequently induced regulatory T cells, to dampen pathogenic responses has burgeoned into a field of immunology into itself. We now know that a large array of mediators can induce a tolerogenic phenotype within dendritic cell populations. These include IL-10 (9, 30, 33, 135–149) and other cytokines (150–158), corticosteroids (143, 159–162), vitamin D3 (160, 163–172), rapamycin (143, 160, 173–175), and neuropeptides (176, 177) (**Table 1**), each of which we will discuss. Although we will not discuss the following populations, it has been reported that dendritic cells can also be rendered tolerogenic by exposure to: anti-CD3 (178); *Aspergillus oryzae* protease (162); aspirin (179); atorvastatin (180); butyric or mycophenolic acids (181); the α7β0 isoform of C4b-binding protein (182); the FasL decoy receptor, decoy receptor-3 (183); galectin-1 (184, 185); growth-related oncogene (GRO)-gamma (186); intravenous immunoglobulin (IVIg) (187, 188); protein kinase C inhibitors (189); or retinoic acid (190–193), or by inhibition of miRNA let-7i (194). IL-10-, vitamin D3-, dexamethasone-, and rapamycin-induced tolerogenic dendritic cells stand out as populations that have had been particularly well-studied in mouse and/or human systems, so we will concentrate our discussions on these cells, with the interested reader referred to the cited reports for these alternate populations. Furthermore, given the potential ethical issues with use of dendritic cells transfected with viruses that express tolerogenic molecules (e.g., IL-10, CTLA4Ig) or that suppress stimulatory molecules (e.g., co-stimulatory, immunostimulatory, or pro-apoptotic molecules, such as CD80, IL-12, or TRAIL, respectively (195)), we will not devote significant discussion to these approaches at this time.

INTERLEUKIN-10-INDUCED REGULATORY DENDRITIC CELLS

As noted, IL-10 was one of the first mediators shown to induce human dendritic cells to adopt a tolerogenic phenotype (8, 9, 122,

Table 1 | Phenotypes of human tolerogenic dendritic cells differentiated using different agents.

Agent	DC	DCreg markers	Effector	Mechanisms of tolerance (outcomes)	Treg induced	Reference
Nil	Immature MDDC	↓ Co-stim, MHCII, IL-12	↓ Co-stim, MHCII	Induction of T cell anergy	N.D.	(119–122)
Apoptot. cells	Immature MDDC	↓ Co-stim, MHCII, IL-12 ↑ TGFβ	↓ Co-stim, MHCII	Induction of T cell anergy	$Foxp3^+$ Treg	(116, 118)
IL-10	Semi-mature MDDC ("DC10")	↓ Co-stim, MHCII, IL-12 ↑ IL-10, ILT-2, -3, and -4, PD-L1 and -L2, GILZ	IL-10 and contact-depend	↓ Autol. T cell prolif.	$CD25^+Foxp3^+$ Treg	(30, 143, 196–198)
	Immature MDDC	↓ Co-stim, MHCII ↑ ILT3, IL-10, GILZ, TLR2	N.D.	↓ Allo. T cell prolif.	N.D.	(136, 158, 164)
	Immature MDDC ("DC-10")	↓ Co-stim, MHCII ↑ ILT-2, -3, -4, HLA-G	IL-10, ILT4, HLA-G	↓ Allo. T cell prolif. ↑ Tr1	Tr1	(33)
Vit D3	Immat. MDDC	↓ Co-stim and CD83, MHCII ↑ HLA-DR	N.D.	↓ Allo. T cell prolif.	Not $CD25^+$ $Foxp3^+$ Treg	(160)
	MDDC + LPS	Intermed co-stim/MHCII ↑ IL-10, TNF, PD-L1 and ILT3	PD-L1	↓ Allo. T cell prolif., Teff > IL-10 Treg	$CD25^+Foxp3^+$ Treg or ND	(163, 170–172, 199)
	MDDC + TLR stim.	hMDDC, LPS maturation	LPS ≫ IL-10 med	↓ Allo. T cell prolif.	N.D.	(164)
	MDDC + LPS	↑ Surface TNF ↓ Secr. TNF	Surface TNF	↑ Treg induction	N.D.	(167)
	Dermal DC	N.D.	IL-10	↓ Allo. T cell prolif.	Tr1 cells	(200)
	Langerhans cells	N.D.	TGFβ	↓ Allo. T cell prolif.	$CD25^+Foxp3^+$ Treg	(200)
	$CD141^-CD1c^+$ blood DC	↑ CD83 ↑ CD141, CD14, ILT3, MØ mann. R	IL-10		$CD25^+Foxp3^+$ Treg	(90)
Dex	Immat. MDDC	↑ CD86, MHCII CD83 med	IL-10	↓ Allo. T cell prolif.	N.D.	(160)
	MDDC + LPS	Intermed co-stim/MHCII ↑ IL-10	N.D.	↓ Allo. T cell prolif., Teff > IL-10 Treg	IL-10-secreting, contact-depend. Treg	(171)
	MDDC ± TLR stim.	Intermed co-stim/MHCII $ILT3^+$, $IL\text{-}10^+$, $GILZ^+$, $TLR2^+$	N.D.	↓ Allo. T cell prolif.		(164)
	DC2.4 cells	↓ IL-12	N.D.	↓ Allo. T cell prolif.	$CD25^+Foxp3^+$ Treg	(201)
Steroid	MDDC	$GILZ^+$	N.D.		N.D.	(202)
VitD3 + Dex	MDDC	↓ Co-stim and CD83, MHCII > CD14, HLA-DR, CD80, CD273	IL-10	↓ Allo. T cell prolif. ↓ $CD25^+Foxp3^+$ Treg ↑ Tr1 and Breg	Tr1 or N.D.	(165, 203)
VIP-DC	Immature MDDC	↓ Co-stim, MHCII ↑ IL-10	N.D.	Weak naïve allo T cell activation	Tr1 and $CD4^+$ $CD28^-CTLA4^+$ Treg	(176, 204)
Rapamycin	Immat. MDDC	↓ Co-stim med. MHCII	IL-10?	↓ Allo. T cell prolif. ↑ $Foxp3^+CD25^+$ Treg	$CD25^+Fopx3^+$ Treg	(160, 205, 206)

135, 136, 141, 148, 150, 209–212). These reports together indicated that IL-10-differentiated monocyte-derived dendritic cells display reduced levels of MHCII and co-stimulatory markers, and can induce Teff cell anergy. Sometime later it was shown that IL-10-induced semi-mature $CD14^+$ monocyte-derived dendritic cells (DC10) from atopic asthmatic individuals suppress specific allergen-driven proliferative and Th2 cytokine responses of autologous peripheral blood $CD4^+CD25^{-/lo}Foxp3^-$ Teff cells, and convert these Teff cells into regulatory T cells (30). The maturation status of these DC10 was attributable to their exposure during differentiation to a stimulatory cocktail containing IL-1β, TNF, IL-6, and PGE2, in addition to IL-10 (30), but these cells are resistant to further, LPS-induced, maturation (209). DC10 express low levels of MHCII, co-stimulatory markers, 4-1BBL and OX40L, but they strongly express DEC205, IFNα1, CCR7, ILT2 (an inhibitory HLA-G receptor), as well as IL-10 (**Table 2**). They induce Teff cells to differentiate into IL-10-secreting $CD25^+Foxp3^+LAG\text{-}3^+CTLA4^+$ regulatory T cells, which in turn suppress allergen-driven responses of autologous Teff cells in a contact-dependent fashion (30). Others found that similar semi-mature IL-10-differentiated dendritic cells express high levels of ILT3, ILT4, PD-L1, and PD-L2, that they (but not immature cells) respond strongly to the lymph node-homing chemokine CCL19 (143), and that they induce regulatory T cells that also suppress allogeneic T cell responses in a contact, but not IL-10- or TGFβ-dependent fashion (196). These DC10 also express glucocorticoid-induced leucine zipper (GILZ), which is both necessary and sufficient for expression of IL-10, ILT3, and PD-L1 by these cells – GILZ silencing eliminates their tolerogenic activities (197, 198). IL-10-differentiated human monocyte-derived dendritic cells that have never been exposed to maturation-inducing agents are also tolerogenic (33, 148, 212). As noted above, a minor population of IL-10-producing circulating dendritic cells, called DC-10, was recently identified in humans (33), and those investigators also generated an analogous population of immature IL-10-differentiated dendritic cells (DC-10) that similarly express IL-10 (**Table 2**), as well as the inhibitory receptors ILT2, ILT3, ILT4, and HLA-G (33). Others have noted that such cells also express signaling lymphocyte activation molecule (SLAMF1, CD150) (148), which inhibits CD40-mediated signal transduction (213), and would therefore interfere with two-way dendritic cell-T cell conversations. These cells have been reported to suppress Teff cell responses in a manner that is contact-dependent, and independent of any role for secreted soluble mediators (148), although others note that IL-10 secretion and cell-surface inhibitory receptors are both important to the regulatory activities of such immature IL-10-differentiated dendritic cells (33). It is very intriguing that exposure of semi-mature human dendritic cells to IL-10 leads to their induction of classical $CD25^+Foxp3^+$ Treg (30, 196), while exposure of immature human dendritic cells to IL-10 leads to induction of $Foxp3^-$ Tr1 cells (33). It will be interesting to determine whether exposure of such immature regulatory dendritic cells to inflammatory (i.e., maturation-promoting) conditions would qualitatively or quantitatively affect their immunobiology.

Murine DC10 can prevent the onset of asthma in experimental mice, as well as reverse the asthmatic phenotype in severely affected animals (137, 138, 140, 214–216), just as do dendritic cells that have been virally transfected to express very high levels of IL-10 (146). These DC10, which are not exposed to maturational stimuli during differentiation, display low levels of cell-surface MCHII and co-stimulatory markers, are avidly phagocytic and chemotactically responsive to MIP-1α, and express elevated levels of IL-10, TGFβ (137, 138, 215), and PD-L1 (Li et al., unpublished observation). They are highly effective therapeutically in mouse models of ovalbumin (OVA) – (138, 140, 214–216) and house dust mite – (137) asthma. In both settings, DC10 abrogate airway hyperresponsiveness (AHR) within 3 weeks of treatment and dampen the allergic Th2 phenotype

Table 2 | Impact of phenotype on the levels of IL-10 secretion by regulatory dendritic cells.

Differentiating agent	DC (IL-10 levels)	Reference
NON-REGULATORY DENDRITIC CELLS		
TNF	Semi-mature MDDC (≈35 pg/ml)	(30)
Nil	semi-mature MDDC (LPS, >700 pg/ml; CD40L, >2 ng/ml)	(207)
TOLEROGENIC DENDRITIC CELLS		
Vitamin D3/dexamethasone	MDDC (9 ng/ml)	(203)
C1Q	MDDC (5 ng/ml)	(208)
Vasoactive intestinal peptide	MDDC (LPS, ≈5 ng/ml)	(176)
Galectin-1	MDDC (LPS, ≈500 pg/ml)	(185)
Vitamin D3	Dermal DC (CD40L, ≈300–700 pg/ml)	(90)
	MDDC (unstim or LPS, ≈100 pg/ml)	(160, 164)
	MDDC (LPS or CD40L, ≈2 ng/ml)	(167)
	MDDC (CD40L, 4 ng/ml)	(163)
IL-10	Immat. MDDC (unstim, 200–750 pg/ml; CD40L, 1.5 ng/ml)	(33, 143, 163, 164)
	Semi-mature MDDC (unstim, 300 pg/ml; LPS, 7 ng/ml)	(30, 158)
Dexamethasone	Immat MDDC (unstim, 25–200 pg/ml)	(143, 159, 160, 198)
	MDDC (LPS or CD40L, 0.5–3 ng/ml)	(159–161, 198)
Rapamycin	MDDC (unstim or LPS, 50–100 pg/ml)	(143, 160)
TGFβ	MDDC (unstim, 200 pg/ml; LPS, ≈2 ng/ml)	(143, 158)

in an allergen-specific fashion (137, 138, 140, 215). This suppression of allergen-induced airway eosinophil and Th2 cytokine responses and circulating allergen-specific IgE and IgG1 levels is progressive, such that at 8 months after a single DC10 treatment these parameters are at near background levels (138), although four DC10 treatments bring the asthma phenotype to near background within 2 months (138). Cell tracking studies indicate that DC10 that are delivered intraperitoneally accumulate maximally in the lungs and lung-draining lymph nodes within 1 week, but few, if any, DC10 can be detected within any anatomic compartment at 3 weeks post-treatment (214). This indicates that while tolerance induced by DC10 is long-lived, most of its impact is realized only after the treatment cells have disappeared from the body. That is consistent with the observation that DC10 treatments induce $CD4^+CD44^{hi}CD69^{hi}CD62L^{lo}CD25^{lo}Foxp3^-$ Teff cells to transdifferentiate into $CD4^+CD25^+Foxp3^+$ Treg, with maximal Treg activation occurring at 3 weeks after DC10 treatment (215). Human DC10-induced $CD25^+Foxp3^+$ Treg express LAG3 and CTLA3 (30), while the analogous Treg in DC10-treated asthmatic mice express LAG3, cytotoxic T lymphocyte antigen-4 (CTLA4) (137, 215), ICOS, PD-1, GITR (215), and neuropilin-1, but lower levels of Helios (217). Infectious tolerance is also evident in these animals, as the endogenous pulmonary $CD11c^+$ dendritic cells of DC10-treated asthmatic animals also take on a regulatory phenotype (Li et al., unpublished observation). While DC10 engage $CD4^+CD25^+Foxp3^+$ natural (n)Treg in a productive fashion and these T cells have a modest role in the asthma tolerance within DC10-treated animals, DC10-induced $CD25^+Foxp3^+$ (i)Treg are many-fold more effective than naturally occurring $CD25^+Foxp3^+$ regulatory T cells (nTreg) of identical TCR specificity in suppressing the asthma phenotype (217).

IL-10 expression by immature or otherwise quiescent dendritic cells has been reported numerous times to be important to tolerance induced by these cells (24, 107, 218), and DC10 (as well as DC-10) express yet higher levels of this regulatory cytokine (30, 33, 137, 138, 140, 215, 216) (**Table 2**). Indeed, expression of IL-10 by DC10 is critical (140, 214) although not sufficient for tolerance induction, inasmuch as MHCII-knock-out DC10, which expresses otherwise therapeutic levels of IL-10, do not induce tolerance (214). Moreover, combined IL-10 and MHCII expression by DC10 is still not sufficient for full expression of tolerance – allergen-presenting CD80/CD86 double knock-out (214) or CD40-knock-out (W. Dawicki, H. Huang and J.R. Gordon, unpublished observations) DC10 still do not induce tolerance at levels equivalent to wild-type DC10 (214). This underscores that conversion of Teff cells to regulatory T cells by DC10 requires not only delivery of tolerogenic signals to the T cell, but also productive feedback from the engaged T cell to the DC10.

VITAMIN D3-INDUCED REGULATORY DENDRITIC CELLS

Vitamin D and its metabolites would appear to have a significant influence within the immune system, such that there is substantial evidence of an unrealized potential for its use in an array of immunologic disorders [reviewed in Ref. (219, 220)]. It is clear that vitamin D3 can induce differentiation of tolerogenic dendritic cells (DC-VitD3) (163, 170, 221, 222). Addition of vitamin D3 to mouse bone marrow (177, 223, 224) or human monocyte-derived (164, 170, 172, 225) dendritic cell cultures induces cells that express low levels of MHC II and co-stimulatory molecules, and produce IL-10 instead of IL-12 (**Table 2**). Semi-mature monocyte-derived DC-VitD3 express augmented levels of TNF and PDL-1, and this PDL-1 is reportedly critical to their induction of IL-10-expressing contact-dependent Treg (171), as is expression of membrane-bound TNF by these dendritic cells (226). As with IL-10-differentiated dendritic cells, DC-VitD3 only respond to the lymph node-homing chemokine CCL19 if they have been exposed to maturational stimuli (143). This again raises the question of whether such chemokine-dependent lymph node homing might reasonably be expected to contribute, if not be critical, to the tolerogenic activities of regulatory dendritic cells. Addition of vitamin D3 to cultures of human skin Langerhans cells leads to expression of TGFβ by these cells and thereby downstream induction of $CD25^{hi}CD127^{lo}Foxp3^+$ cells (i.e., classical inducible Treg) (200). It similarly induces $CD141^-CD1c^+$ human blood dendritic cells to differentiate into IL-10-expressing dermal dendritic cell-like $CD141^+CD14^+ILT3^+$ cells that induce development of $CD25^{hi}CTLA4^+Foxp3^+$ Treg responses (90). In contrast, addition of vitamin D3 to cultures of human dermal dendritic cells upregulates expression of IL-10 and their induction of IL-10-expressing $Foxp3^-$ Tr1 cells (200). This highlights again that exposure of different dendritic cell populations to the same mediator can have very divergent outcomes in terms of the type(s) of regulatory T cells so induced. DC-VitD3 have been shown to be tolerogenic *in vivo* as well. Treatment of diabetic mice with pancreatic islet antigen-pulsed DC-VitD3 prior to pancreatic islet transplantation significantly decreases subsequent islet rejection (166), while sensitization of mice with H-Y antigen-pulsed DC-VitD3 leads to prolongation of male skin grafts in female recipients (177, 223, 224).

DEXAMETHASONE-INDUCED TOLEROGENIC DENDRITIC CELLS

The anti-inflammatory and immunosuppressive properties of corticosteroids have been known and employed clinically since their discovery some 75 years ago (227). While glucocorticoid treatments have significant clinical benefits in terms of suppressing inflammation, and it has been shown that they increase the numbers of $CD4^+CD25^{hi}$ cells and Foxp3 expression levels in multiple inflammatory settings, these increases are not necessarily associated with augmented Treg activity (228). Corticosteroids do induce immature dendritic cells to adopt a tolerogenic phenotype and thereby contribute to the anti-inflammatory properties of these agents (159, 229, 230), but the fact that mature dendritic cells undergo apoptosis in response to *in vitro* or *in vivo* dexamethasone treatment suggests that its effects on dendritic cells are somewhat more complex (231). Dendritic cells that are differentiated in the presence of dexamethasone (DC-Dex) express low levels of co-stimulatory markers and MHC II, produce elevated levels of IL-10 and less IL-12 (159, 161, 164, 171, 229, 230, 232), and express modestly elevated levels of ILT2 (198) and ILT3, but high levels of GILZ (164). As with semi-mature IL-10-differentiated dendritic cells, GILZ expression by DC-Dex is critical to their expression of IL-10, ILT3, and B7-H1/PDL-1 (197); both populations also maintain their immunosuppressive phenotype even after stimulation with TLR4 agonists (209, 233, 234).

The similarities between DC10 and dexamethasone-conditioned dendritic cells extends further – dexamethasone-exposed DC2.4 dendritic cells also induce Foxp3$^+$ Treg differentiation *in vitro* (201), while use of DC-Dex immunotherapy for experimental corneal allografts similarly leads to increased tissue levels of intragraft Foxp3$^+$ T cells, reduced levels of graft inflammatory cell infiltrates, and prolonged graft survival (235). And others have reported that repetitive stimulation of T cells with DC-Dex induces the T cells to adopt a contact-dependent regulatory T cell phenotype (171). DC-Dex treatment of murine recipients of MHC-mismatched heart transplants leads to delayed rejection of the allografts (234) although, oddly, DC-Dex treatments reportedly accelerate antibody-mediated graft rejection responses to transplanted MHC-mismatched pancreatic islets in rats (236). Interestingly, the contact-dependent regulatory T cells induced by DC-Dex, but not those induced by DC-VitD3, reportedly suppress T cell responses in an antigen-independent fashion (171), although others have shown that, as a general feature, activated regulatory T cells readily suppress by-stander Teff cell responses (237, 238).

VITAMIN D3 AND DEXAMETHASONE-INDUCED TOLEROGENIC DENDRITIC CELLS

While vitamin D3 and dexamethasone each can induce a tolerogenic phenotype in dendritic cells, some investigators have further assessed the regulatory activities of cells generated in the presence of both vitamin D3 and dexamethasone (DC-Dex/VitD3). DC-Dex/VitD3 produce much high levels of IL-10 (i.e., 9 ng/ml) (203) than either DC-VitD3 or DC-Dex (i.e., 0.1–4 ng/ml) (**Table 2**) (143, 159, 160, 163, 164, 167, 198), and thus display a higher IL-10/IL-12 expression ratio and poorly stimulate allogeneic T cell proliferation responses (165). They reportedly cannot effectively prime naïve CD8 T cells but, interestingly, while a single DC-Dex/VitD3 treatment drives expansion of memory CD8 T cells, any subsequent DC-Dex/VitD3 exposure leads to collapse of the CD8$^+$ T cell populations (239). DC-Dex/VitD3 have been shown to be somewhat effective in suppressing colitis pathology in a mouse model, apparently also in an antigen-independent manner (240).

NEUROPEPTIDE-INDUCED TOLEROGENIC DENDRITIC CELLS

Vasoactive intestinal peptide (VIP) is a 28-amino acid immunomodulatory neuropeptide that binds to B-class G-protein-coupled receptors such as the VPAC1 and VPAC2 (241, 242). VIP treatments induce regulatory T cell responses in experimental animals and with human Teff cells (243). For example, VIP treatment of mice with TNBS-induced colitis induces tolerance responses, dampening TLR2- and TLR4-induced inflammation and increasing expression of Foxp3 and TGFβ (244), as it does in a rat model of collagen-induced arthritis (245). But VIP can act directly on the Teff cells – culture of CD25$^-$Foxp3$^-$ Teff with VIP induces their differentiation into CD25$^+$Foxp3$^+$ Treg that express high levels of IL-10 and CTLA4 and are protective in a mouse model of graft versus host disease (GVHD) (246). Nevertheless, VIP can also induce dendritic cells to adopt a regulatory phenotype and thereby affect tolerance by this means. Differentiation of human dendritic cells in the presence of VIP (DC-VIP) or the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) induces the development of cells that secrete of high levels of IL-10, and strongly induce regulatory T cell responses. DC-VIP treatments dampen pathology in a number of experimental settings, including experimental allergic encephalomyelitis (EAE), rheumatoid arthritis (247), bone marrow transplant-induced GVHD (248), and colitis (249). While a number of reports indicate that DC-VIP induce Tr1 phenotype regulatory cells, as determined by secretion of IL-10/TGFβ, but not IFNγ, IL-2, IL-4, or IL-5 (204, 250–252), other reports indicate that DC-VIP instead induce CD4$^+$CD25$^+$Foxp3$^+$ Treg responses (253–255) in some of the same model systems. DC-VIP can also induce IL-10-secreting CD28$^-$CTLA4$^+$CD8$^+$ Treg (176, 252). VIP-secreting VIP-lentivirus-transfected DC are similarly tolerogenic in mouse models of acute and chronic EAE and cecal ligation-and-puncture sepsis (177). It has been speculated that DC-VIP would be more effective therapeutically when targeting Th1 rather than Th2 responses (176), ostensibly because VIP skews Th1 or Th17 T cells to a Th2 phenotype (256). This raises an important question in dendritic cell immunotherapeutics, and that is whether the specific type of regulatory cell to be employed (e.g., DC-Dex versus DC-VIP) needs to be carefully matched with, for example, the Th1, Th2, or Th17 nature of the target disease in order to ensure optimized outcomes.

RAPAMYCIN-INDUCED TOLEROGENIC DENDRITIC CELLS

Rapamycin is a macrolide immunosuppressive agent that dampens dendritic cell maturation through binding to the serine/threonine protein kinase mammalian target of rapamycin (mTOR). Signaling via mTOR has broad-ranging effects in many systems, including the nervous system, nutrition, and others, where it regulates cell growth, proliferation, motility, and survival (257). Antigen recognition by naïve CD4$^+$ and CD8$^+$ T cells activates mTOR and thereby fosters cellular progression to a committed Foxp3$^-$ Teff phenotype (205), while suppression of mTOR with rapamycin leads instead to induction of fully functional CD25$^+$Foxp3$^+$ Treg (258). Thus it was reported some time ago that rapamycin increases the regulatory activities of CD4$^+$CD25$^+$Foxp3$^+$ Treg (206). Clinically, rapamycin has been widely used to prevent allograft rejection, particularly in renal transplant patients (206), although the potential for rapamycin-related adverse cutaneous manifestations in these patients has limited its broad applicability (259). Rapamycin affects both T cells and dendritic cells, although it displays divergent effects on myeloid and monocyte-derived dendritic cells, augmenting the allostimulatory capacity of the former cells but markedly dampening the immunostimulatory phenotype of monocyte-derived dendritic cells (260). In experimental systems rapamycin treatments impair Flt3L mobilization of murine dendritic cells, their upregulation of co-stimulatory molecule and inflammatory cytokine expression, and their allostimulatory activity (261), even after exposure to activating agents such as LPS or anti-CD40 (262). Mouse dendritic cells that are differentiated in the presence of rapamycin (DC-Rap) induce naïve T cells to differentiate into CD25$^+$Foxp3$^+$ Treg (263). Moreover, such DC-Rap enhance apoptotic death among alloreactive CD8 T cells (264), further contributing to the tolerance response of transplant recipients. DC-Rap treatment of murine heart transplant recipients similarly induces outgrowth within the transplants of Foxp3$^+$ Treg and, as a consequence, long term organ survival (262), just

as has been shown in numerous other studies (173, 206, 261, 262, 265, 266).

IMPACT OF DELIVERY ROUTE AND INFLAMMATION ON THERAPEUTIC OUTCOMES

SELECTING THE CORRECT DELIVERY ROUTE FOR TOLEROGENIC DENDRITIC CELLS

Not all routes for delivery of tolerogenic dendritic cells will necessarily provide the desired outcomes. For example, we reported that CD45.2$^+$ DC10 that are delivered intraperitoneally to congenic CD45.1$^+$ mice with a severe asthma phenotype appear within the lungs and airways of recipient mice within 2 days of delivery, achieve maximal numbers in this compartment by 7 days and then wane thereafter. DC10 appear in the lung-draining (mediastinal) lymph node of these animals in lower numbers, but with approximately the same kinetics, and also in the spleen but not cervical nodes, MLNs, blood, bone marrow, or liver. Within 3 weeks of delivery the treatment cells are no longer detectable in the lungs or mediastinal lymph nodes (214), suggesting that the natural lifespan of such DC10 may be 2–3 weeks. We know that DC10 treatments correct ~50% of the pathognomic bronchial hyperresponsiveness seen in asthma phenotype mice within 2 weeks of treatment and that by 3 weeks this airway response is completely normalized (138). Moreover, the time of maximal activation of regulatory T cells in the lungs of these animals is 3 weeks after DC10 delivery, but it was not determined whether the primary site within which the DC10 induce Teff cells to differentiate into regulatory T cells was *in situ* in the lungs or in the mediastinal lymph nodes (or both) (215). This remains an important, but unanswered question.

We also assessed the relative effects of intraperitoneal (i.p.), transtracheal (t.t.), subcutaneous (back skin; s.c.), or intravenous (i.v.) DC10 delivery to asthmatic animals and found that i.p. or t.t. delivery were equally effective, fully reversing bronchial hyperresponsiveness, and rapidly dampening airway eosinophil and Th2 cytokine responses to allergen challenge and circulating allergen-specific IgE and IgG1 levels (138). The s.c. DC10 treatments dampened the airway recall responses to allergen challenge, but not bronchial hyperresponsiveness, nor did they significantly reduce systemic IgE levels (138). On the other hand, multiple investigators have reported that s.c. delivery of tolerogenic dendritic cells is protective in rat models of EAE (267–271), which suggests that the anatomic site of the target pathology in immunotherapeutic applications may be important in selecting the delivery route for the treatment dendritic cells. Intravenous delivery of DC10 has no discernible impact of the disease phenotype in a mouse model of asthma (138, 272) or a rat model of EAE (271), but in mouse models of cardiomyopathy (147), experimental immune myocarditis (149, 273), and diabetes (274, 275) i.v. delivery of tolerogenic dendritic cells significantly reduces local pathology and induces tolerance. Similarly, i.v. infusion of DC-VitD/IL-10 in a rhesus macaque model of allogeneic kidney transplantation significantly prolonged survival relative to control animals (rapamycin/CTLA4Ig treatment, but no dendritic cells) (276). There has not been a sufficient number of comprehensive studies on the impact of the route of dendritic cell delivery on tolerance outcomes to generate specific guidelines at this point in time, but it does appear that the disease or compartment being targeted may be an important consideration. Certainly, we would expect that the cells should be migration-competent (i.e., express appropriate chemokine receptors), such that they are able to travel to the disease target site or its draining lymph nodes in order to best interact with the cognate Teff cells.

USE OF TOLEROGENIC DENDRITIC CELLS IN INFLAMMATORY SETTINGS

An important consideration in clinical use of tolerogenic dendritic cells, particularly when targeting inflammatory diseases (e.g., colitis, inflammatory bowel disease), is whether pre-existing adverse conditions that these cells might encounter after delivery can alter or ablate their tolerogenic activity. If so, could an inflammatory milieu convert the treatment dendritic cells into immunostimulatory populations that might exacerbate rather than ameliorate disease severity? While immature dendritic cells can have substantial tolerogenic activities, we know that exposure of these cells (133, 134) or even some populations of semi-mature dendritic cells (133, 134) to inflammatory environments can induce them to differentiate into potently immunostimulatory cells that *augment* disease severity. With this in mind, many investigators have assessed the impact of maturation-provoking (30, 90, 143, 165, 197) or otherwise inflammatory (163, 164, 167, 170, 171, 177, 222) signals on the tolerogenic phenotype of their differentiated dendritic cells. Dendritic cells express receptors for and can be activated by a number of pro-inflammatory cytokines (e.g., IL-1, TNF, IFN, TSLP) (277) and they can express numerous pattern recognition receptors [e.g., protease-activated receptors (PARs), TLR, C-type lectin receptors (78, 164, 278–281)], retinoic acid-inducible gene-1 (RIG-1) and the melanoma differentiation-associated gene-5 (MDA-5) (281), through which they interact with microbial and non-microbial agents. For example, a number of "natural" allergens (e.g., house dust mite) trigger inflammatory responses through their abilities to activate cells via PAR2 (282, 283) or C-type lectin receptors such as DC-SIGN and dectin-2 (284), while TLR signaling can potently activate expression of inflammatory signals by immature or mature dendritic cells. There have been a number of excellent reviews that address the expression of TLR by human and mouse dendritic cells [e.g., Ref. (281)], such that we will not address this issue herein.

Toll-like receptor signaling within tolerogenic dendritic cell populations does not always have a detrimental outcome. For example, BDCA-1$^+$ human liver dendritic cells secrete substantial amounts of IL-10 on TLR ligation, and this contributes to their high level induction of CD25$^+$Foxp3$^+$ Treg (11). Human DC10, DC-Dex, and DC-VitD express the same panel of TLR as monocyte-derived dendritic cells, such that all are responsive to Pam3CSK4, polyinosinic-polycytidylic acid, LPS, and flagellin (164), but the tolerogenic populations uniquely upregulate expression of TLR2 on TLR engagement (164). Moreover, TLR2 or TLR4 signaling in human DC-VitD3 and DC-Dex induces expression of the tolerance-promoting cytokines IL-10 and IL-27 (160, 285). Others have reported that human DC-Dex are refractory to challenge with an array of heat-killed gram-negative bacteria (e.g., *Escherichia coli*, *Protheus mirabillis*, *Klebsiella pneumoniae*, *Salmonella thyphimurium*) (286), while DC-Rap (160) and DC-VIP (252) are resistant to reversal of their tolerogenic phenotype by

LPS challenge. Interestingly, while isolated LPS challenge induces an IL-12 response by immature monocyte-derived dendritic cells, simultaneous exposure of these cells to LPS and IFNγ reportedly leads to a transient IL-12 response that is replaced within 24–48 h with a robust IL-10 response (287).

Finally, while we may well be able to design and generate tolerogenic dendritic cells that are resistant to reversal of phenotype by inflammatory environments, it is clear that the tolerance they induce is also dependent on transference of that phenotype to the regulatory T cells with which they interact. Moreover, infectious tolerance also involves the conversion of endogenous tissue dendritic cells into tolerogenic populations by the induced regulatory T cells (226, 288). Indeed, it has been suggested that a defect in such infectious tolerance processes may contribute to the development of an asthma phenotype in affected individuals (289). The desired outcome in dendritic cell immunotherapy is the induction of regulatory T cells that can reverse pathogenic Teff cell responses, but at least some populations of regulatory T cells can be converted into pathogenic Teff cells in the context of inflammatory environments – it has been shown that $Foxp3^+$ Treg can convert to Th17 cells in animals with colitis (290, 291), but we seem to have only scant evidence regarding the extent to which other populations of regulatory cells (e.g., Tr1 or Th3 cells) can be enticed to such reversal of phenoytpe *in vivo*. In considering whether inflammatory environments may differentially affect the phenotype of regulatory T cells (or dendritic cells), we query whether the regulatory T cells that are naturally associated with a specific compartment (e.g., Th3 cells in the gut) might be more resistant to reversal of phenotype by challenges they would routinely encounter in that compartment than other regulatory T cells (e.g., Treg, Tr1). Finally, we raise the issue of whether in some specific settings, it might be advisable to activate multiple types of regulatory T cell responses, such that the tolerance so induced might be less susceptible by reversal by subsequent coincidental inflammatory events.

CLINICAL APPLICATION OF TOLEROGENIC DENDRITIC CELLS

The first tolerogenic dendritic cell study in humans was undertaken by Ralph Steinman's lab. They demonstrated that s.c. administration of antigen-loaded immature dendritic cells (2×10^6 cells/subject) was well tolerated by the study subjects and also that the treatments could suppress antigen-specific $CD8^+$ T cell responses (128) for ≤6 months (127). More recently a clinical trial was undertaken with 10 subjects with type 1 diabetes, each of whom was given 1×10^7 autologous dendritic cells intradermally four times at 2 week intervals; the treatment cells had been transduced with anti-sense oligonucleotides to silence co-stimulatory molecules (i.e., CD40, CD80, and CD86), although efficacy data on that silencing was not reported (292). The authors had developed their silencing protocols in a mouse model of type 1 diabetes and shown that the dendritic cell treatments had had statistically significant, though quite modest, disease-sparing effects (293). As with the earlier study by Steinman (127, 128), there were no adverse events related to the dendritic cell treatments in this latter study, but there were few if any immunologically discernible tolerance outcomes attributable to the dendritic cell treatments (292).

There have been a large number of *in vitro* studies performed as proof of principle that tolerogenic dendritic cells can efficiently reduce Teff cell responses in humans. As noted above, it was shown that semi-mature IL-10-differentiated dendritic cells (i.e., DC10) generated from atopic asthmatic donors can suppress the responses of autologous T cell to specific allergen. Moreover, the DC10 induce the outgrowth of immunosuppressive $CD4^+CD25^+Foxp3^+LAG3^+CTLA4^+$ Treg from the peripheral blood Teff cell pool (30). Others have reported that DC-VitD/Dex from individuals with rheumatoid arthritis (294) or DC-VitD3 from subjects with relapsing-remitting multiple sclerosis (295) are both able to suppress autologous $CD4^+$ Teff cell responses to specific antigen-presenting mature dendritic cells.

In conclusion, it is clear that multiple mediators can induce a tolerogenic phenotype in dendritic cells, and that these substantially influence the conversations that occur between the dendritic cell and naive or Teff cells. These tolerogenic dendritic cells employ both secreted mediators (e.g., IL-10, retinoic acid) and inhibitory receptors to drive regulatory T cell induction, but can also provide additional signals (e.g., integrins) to direct these nascent Treg to the appropriate anatomic compartment (**Figure 1**). A major challenge we will face in the application of such tolerogenic dendritic cells for immunotherapy will be to carefully match or optimize the type(s) of tolerogenic dendritic cells to be employed with the clinical targets and desired endpoints.

AUTHOR CONTRIBUTIONS

Wojciech Dawicki reviewed the literature for and wrote parts of the section on naturally occurring populations of dendritic cells, Yanna Ma contributed to the section on T cell biology, Laura Churchman wrote the introduction, Sara A. Gordon collated the literature on the different types of dendritic cells that have been reported, and John R. Gordon wrote the section on the different types of experimental dendritic cells that have been reported. All authors contributed to the planning and editorial phases of the review.

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Therapeutic use of dendritic cells to promote the extranodal priming of anti-tumor immunity

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Ectopic lymphoid tissue, also known as tertiary lymphoid organs (TLO) develop adaptively within sites of chronic tissue inflammation, thereby allowing the host to efficiently crossprime specific immune effector cells within sites of disease. Recent evidence suggests that the presence of TLO in the tumor microenvironment (TME) predicts better overall survival. We will discuss the relevance of extranodal T cell priming within the TME as a means to effectively promote anti-tumor immunity and the strategic use of dendritic cell (DC)-based therapies to reinforce this clinically preferred process in the cancer-bearing host.

Keywords: dendritic cells, extranodal, cross-priming, therapy, cancer

INTRODUCTION

In the classical model of peripheral T cell activation, tissue-resident dendritic cells (DCs) capture antigens (such as foreign pathogens, tumor cell debris, etc.) in an inflammatory microenvironment, leading to the migration of antigen-laden $CCR7^+$ DC to regional draining lymph nodes [LN; aka secondary lymphoid organs (SLO)], where activation of cognate T cells occurs (1–3). After appropriate proliferative expansion and maturation, T effector cells may then enter the blood circulation and be recruited into tissue sites where they are competent to recognize and react against relevant antigen-presenting cells, such as virally infected host cells or tumor cells (4). Recent evidence obtained in a range of translational and clinical models suggests, however, that this classical/conventional paradigm may be operationally overly simplistic, and that extranodal (cross)priming of antigen-specific T cells can occur in peripheral tissues, often times in conditionally established tertiary lymphoid organs (TLO) (5–9).

SLO/TLO DEVELOPMENT: NATURAL AND INDUCED

The developmental formation of SLO is believed to require the interaction of so-called lymphoid tissue inducer cells (LTi) bearing a $CD3^-CD4^+CD45^+IL\text{-}7R^+c\text{-}Kit^+$ phenotype that produce lymphotoxin α/β [LTα/β; Ref. (10, 11)] with $LT\beta R^+$ stromal "organizer" cell populations that may derive from adipocyte precursors (12), leading to corollary stromal cell elaboration of the SLO homeostatic chemokines CCL19, CCL21, and CXCL13 (8, 9, 13–15). These chemokines sustain recruitment of LTi and other lymphocytes into SLO, resulting in the development of a mature lymphoid organ architecture [i.e., based on the formation of follicular structures containing B cells and surrounding "cortical" zones that are diffusely populated by $CD4^+$ and $CD8^+$ T cells, antigen-presenting cells (including $CD11c^+$ DC), and $PNAd^+$ high-endothelial venules (HEV; (8, 15–19))].

Naive ($CD62L^+CCR7^+$) T cells enter SLO via interaction with $PNAd^+$ HEV which are "decorated" with the CCR7 ligand chemokines CCL19 and CCL21 on their luminal surface, thereby facilitating lymphocyte extravasation/directed motility from the blood into the lymph node (20). Of these two chemokines, CCL21 may play the more dominant role in recruiting naïve lymphocytes into SLO, while CCL19 may be differentially cytoprotective in sustaining nodal populations of lymphocytes (20–22). Prolonged CCR7-mediated signaling into recruited T cells, leads to intrinsic upregulation of the sphingosine-1 phosphate receptor 1, EDG1 (23), which is involved in the ultimate departure of primed T cell populations from SLO into the peripheral blood circulation (24, 25).

While classical SLO are encapsulated structures that develop in predictable locations as a consequence of normal immune system development, under pathologic conditions, ectopic lymphoid tissues (aka TLO) may develop in peripheral tissue sites of chronic inflammation (13, 26). TLO formation has been reported within inflamed organs of patients with rheumatoid arthritis (27–29), psoriatic arthritis (30), diabetes mellitus (31–33), autoimmune gastritis [AIG; Ref. (32)], juvenile dermatomyositis (34), and Sjögren's syndrome (35), among others. TLO formation has also been identified in the lungs of influenza virus-infected mice (36), the livers of hepatitis C virus (HCV)-infected patients (37) and in the stomachs of patients infected with *Helicobacter pylori* (38). "Dysfunctional" human lung allografts exhibiting chronic inflammatory responses have also been found to commonly contain TLO (17).

Furthermore, a burgeoning literature supports tumor-associated TLO as important sites of extranodal T cell priming

and epitope spreading in the responder T cell repertoire (13, 39). TLO have been identified in a subset of human melanoma lesions, in which mature DC were found to maintain intimate contact with recruited T cell populations, consistent with the notion of operational extranodal (cross)priming within the tumor microenvironment (TME) (40, 41). Similar results have been reported for murine melanoma models (7, 8). In line with this model, naïve lymphocytes have been identified in TLO within pulmonary lesions of patients with lung cancer, making it likely that these immune cells encounter their cognate antigen for the first time and develop into antigen-specific T effector cells within the TME *in vivo* (16, 42). TLO featuring DC/Type-1 T cell clusters proximal to B cell "nests" have also been identified in human non-small-cell lung cancer specimens (43). In such instances, the density of mature DC found in TLO appeared to be associated with improved long term survival (6, 43). In a subset of patients with breast cancer, HEV have been found in close proximity to $LT\beta^+LAMP^+$ DC in association with profound B/T cell infiltrates in the TME and a more favorable clinical outcome (44). Furthermore, Mulé and colleagues have recently performed a metagene analysis on human (Stage IV, non-locoregional) melanoma metastases and identified a 12-chemokine gene signature (i.e., CCL2, CCL3, CCL4, CCL5, CCL8, CCL18, CCL19, CCL21, CXCL9, CXCL10, CXCL11, CXCL13) correlating with the presence of TLO (containing $CD20^+$ B cell follicles with prominent areas of $CD4^+$ and $CD8^+$ T cells, but not $FoxP3^+$ T_{reg} cells), with better overall survival noted in the TLO^+ subset of patients (41). In a similar vein, Gu-Trantien et al. (45) have also recently observed that the presence of breast cancer infiltrating follicular $CD4^+$ T helper cells (Tfh; expressing CD200, FBLN7, ICOS, SGPP2, SH2D1A, TIGIT, and PDCD1/PD-1, and producing the CXCL13 chemokine) may be directly correlated with; (i) the degree of tumor-infiltrating lymphocytes (TIL), (ii) the formation of TLO-like structures in cancer tissue, and (iii) improved patient clinical response to pre-operative chemotherapy and/or post-surgical disease-free survival.

The conditional formation of TLO in peripheral tissues appears to require the coordinate participation of a similar cast of cellular participants, soluble mediators, and signaling pathways associated with the orchestration of SLO development (14, 15). Ectopic delivery of LTα/β or LIGHT (aka TNFSF-14 or CD258) promotes $PNAd^+$ HEV, CCL19/CCL21 production, massive naïve T cell infiltration, and (tumor-specific) cross-priming in the context of TLO structures (9, 18, 36, 46–49). For example, targeted therapeutic delivery of LTα into the TME via the administration of a fusion protein encompassing the LTα molecule linked to an antibody recognizing a tumor plasma membrane-associated disialoganglioside GD2 (i.e., ch14.18-LTα) resulted in slowed tumor progression and the establishment of mature TLO structures within 9 days of treatment initiation (8). The LTβR ligands LTα/β and LIGHT appear to act directly on endothelial cells and DC in activating NFκB and promoting the expression of adhesion molecules, such as PNAd, VCAM-1, E-selectin, and ICAM-1 by HEV and IL-12p70 production from DC (50–52). In particular, LIGHT is essential for DC-mediated cross-priming of antigen-specific Type-1 T cells (53). Indeed, ectopic expression of LIGHT in the TME elicits profound infiltration and cross-priming of naïve anti-tumor T cells in concert with upregulated stromal cell production of TLO-associated chemokines (CCL21, CXCL9, CXCL10, and CXCL13), increased expression of vascular adhesion molecules (MAdCAM-1, VCAM-1, PNAd), and the presence of mature DC within the TME (9). Interestingly, DC, natural killer (NK) cells, and even B cells can serve as LTα/β producers in pro-inflammatory environments, allowing for the establishment of an autocrine feed-forward loop promoting TLO development in peripheral tissues (36, 54–59). Consistent with these findings noted for pro-TLO immunobiology of LTβR ligands, blockade of the LTβR precludes formation of TLO *in vivo* (60).

In a similar manner, induced expression or ectopic delivery of LTβR downstream mediators, CCL19 or CCL21, in the TME results in inhibition of tumor growth or complete rejection of established tumors associated with increased infiltration by $CD3^+CCR7^+$ T cells and/or DCs in a range of cancer models (32, 61–70). Interestingly, these interventional maneuvers may also reduce frequencies of tumor-associated immunosuppressive T_{reg} cells and MDSC (61).

During the ontogeny of TLO in peripheral tissues, lymphatic vessels (i.e., $PNAd^+$, $MAdCAM\text{-}1^+$, $LYVE\text{-}1^+$, and/or $Prox\text{-}1^+$ HEV) and disorganized clusters of APC and infiltrating lymphocytes appear in advance of canonical mature lymphoid organ architecture characterized by B cell follicular regions (19, 71). Signals that instigate the diffuse-to-organized structural transition of TLO may be provided by cognate T cell recognition of relevant target cell populations within nascent TLO (15, 72). It is important to note, however, that immature TLO have been oft-associated with locoregional immune sequelae including manifestations of autoimmunity and anti-tumor efficacy (5, 32, 71). Hence, while mature TLO may ultimately develop in the chronic disease setting, clinical meaningful immunobiology occurs in advance of such structural developments.

THERAPEUTIC PROMOTION OF TLO

If the formation of TLO allows for extranodal (cross)priming of antigen-specific T cells that are linked to disease pathogenesis (i.e., autoimmunity) or resolution (i.e., infectious disease, cancer), then means by which to prevent or enhance TLO development, respectively, in affected tissues would be anticipated to impact clinical outcome. Perhaps the most strategically simple means by which to apply this paradigm in the cancer setting reflects the implantation of SLO/TLO directly into the TME. Recently, scaffold-based lymphoid tissue engineering has been developed as a means to transplant "intact" TLO directly into tumors in order to affect clinical benefit (73). A previously mentioned alternative to this strategy is clearly the delivery of the LTβR ligands LTα, LTβ, or LIGHT, agonist anti-LTβR antibodies or downstream TLO-associated chemokines (CCL19, CCL21, CXCL13) via protein-based or genetic therapy in order to instigate the locoregional development of TLO in the TME leading to inhibition of tumor growth *in vivo* and extended overall survival (8, 9, 48, 74, 75).

USE OF DC-BASED THERAPY TO PROMOTE EXTRANODAL PRIMING OF ANTI-TUMOR T CELLS

It also appears that the administration of appropriately activated/engineered DC is sufficient to nucleate and/or maintain the

development of TLO *in vivo* (36, 72). For instance, mice vaccinated sub-cutaneously with syngenic DC loaded with apoptotic/necrotic B16 melanoma cell debris develop operational TLO [pseudocapsule; PNAd$^+$ vascular endothelial cells (VEC), T cell/DC infiltrates] at sites of injection, leading to the activation of protective anti-tumor immunity (72). DC genetically engineered to secrete high-levels of CCL21 (DC.CCL21) and injected directly into B16 murine melanomas promote strong extranodal T cell cross-priming/recruitment into the TME, even in LTα −/− mice that lack SLO (8, 68). The superiority of DC.CCL21 in enhancing the cross-priming of protective Type-1 anti-tumor T cell responses has also been confirmed in alternate murine models (76, 77).

In our recent paper (5), we noted that DC engineered to express the Type-1 transactivator protein T-bet (DC.Tbet) and injected directly into sarcomas growing progressively in C57BL/6 mice, led to the cross-priming of protective immunity that was independent of host CD11c$^+$ or BATF3$^+$ DC or the ability of the injected DC.Tbet to migrate to SLO. Instead, we detected the rapid recruitment of NK cells and naïve T cells into the TME within 48 h of DC.Tbet administration, and the differentiation of these TIL into Type-1 effector cells *in situ* within the TME. As shown in **Figure 1**, we observed a diffuse but interactive collection of CD11c$^+$ DC and Tbet$^+$ cells [including both T cells (5) and B220$^+$ B cells] within the TME of MCA205 sarcomas by 48 h post-treatment with DC.Tbet, but not control DC. PNAd$^+$ HEV were not evident at this early time point, but were readily imaged in proximity to large DC-Tbet$^+$ lymphocyte clusters by 5 days post-treatment with DC.Tbet (but not control DC). These data suggest that extranodal priming of protective immunity using therapeutic DC delivery occurs in advance of the formal adoption of classical TLO anatomic structures within the TME (**Figure 2**), and that indeed, the development of such Type-1 cognate immunity (and its pro-inflammatory signals) in the TME may be required for subsequent evolution of mature TLO formatting, as described by Schrama et al. (8). Interestingly, a gene array analysis of DC.Tbet versus control DC did not reveal any striking differences in expression of LTA, LTB, LIGHT, CCL19, CCL21, or CXCL13 mRNA transcripts, suggesting a potentially novel mechanism associated with early TLO development triggered by this DC-based therapy [(5) and Chen, unpublished data]. In this regard, we noted a striking enhancement in DC.Tbet production of IL-36γ/IL-1F9 (>34-fold; Chen, unpublished data). IL-36γ is a novel IL-1 family member cytokine that has been previously reported to be a potent recruiter and activator of naïve T cells that develop strong Type-1 functional polarity (78, 79). As in the case of LTβR ligands, IL-36 also triggers NFκB activation in IL-36R$^+$ DC (79–82), which may prove pivotal for autocrine potentiation of Type-1 DC function and a pro-TLO TME. Whether tumor-associated VEC express IL-36R and activated NFκb in response to IL-36 remains an unanswered question. We are currently evaluating the impact of IL-36γ knock-down in DC.Tbet in order to determine the direct relevance of IL-36γ in the development of TLO and protective immunity in the TME of mice treated with intratumoral administration of DC.Tbet.

SUMMARY AND FUTURE PERSPECTIVES

In the cancer setting, the ability of the host to develop ectopic lymphoid organs (TLO) within or proximal to sites of active disease appears to represent a positive prognostic factor for overall patient survival. TLO represent a regional "factory" in which naïve T cells (and B cells) may be cross-primed by tumor-resident antigen-presenting cells, such as DC, leading to poly-specific adaptive immunity that may limit disease progression and conceivably metastatic spread. By limiting the need for antigen-loaded DC to

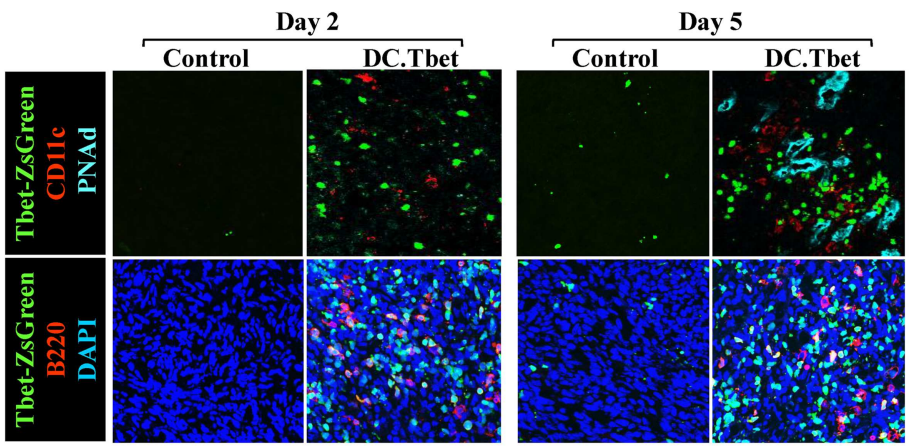

FIGURE 1 | Intratumoral administration of dendritic cells engineered to express T-bet/TBX21 (DC.Tbet) promote the rapid infiltration of Type-1-polarized lymphocytes and dendritic cells and the development of PNAd$^+$ endothelial cells (i.e., HEV). Tbet-ZsGreen Tg mice were injected sub-cutaneously with syngenic MCA205 sarcoma cells and tumors allowed to progressively grow for 7 days, at which time control DC (Control) or DC engineered with recombinant adenovirus to express murine T-bet cDNA were inoculated directly into tumors, as previously described (5). Two or 5 days after DC injection, the mice were euthanized and tumor sections evaluated by fluorescence microscopy for expression of Tbet-ZsGreen protein, CD11c (a marker of DC), B220 (a B cell marker), and PNAd (i.e., Peripheral lymph Node Addressin; a high endothelial venule (HEV) cell marker). PNAd$^+$ HEV were not observed by 2 days post-treatment, but became prevalent by 5 days post-injection of DC.Tbet cells. B, T, and NK cell infiltrates into DC.Tbet [Figure 1 and (5)] exhibited a diffuse distribution pattern in day 2 and day 5 DC.Tbet-treated tumors. Type-1 polarity in infiltrating cells is denoted by nuclear expression of Tbet-ZsGreen. Data are representative of images obtained in three independent experiments performed.

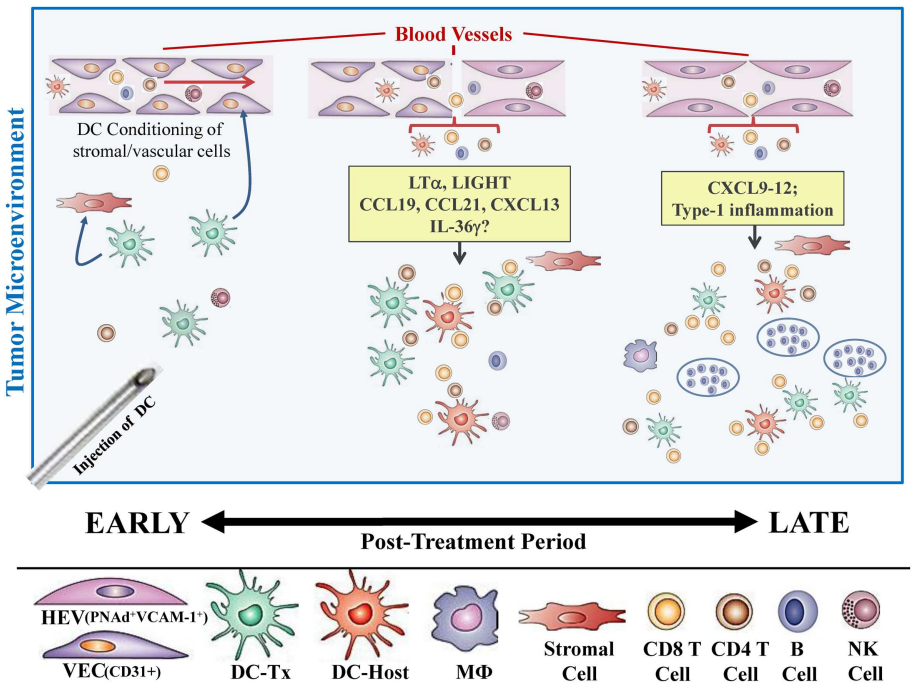

FIGURE 2 | Hypothetical paradigm for extranodal priming of T cells after intratumoral administration of DC.Tbet cells. Injection of DC.Tbet (but not control DC) into the TME leads to the conditioning of tumor-associated stromal cells and vascular endothelial cells (VEC), resulting in stromal cell production of chemokines recruiting naïve leukocytes (B, T, NK cells) and VEC expression of adhesion molecules, such as VCAM-1, as early as day 2 post-treatment [Figure 1 and (5)]. Recruited lymphocytes are assembled in diffuse patterns around CD11c$^+$ (both injected and host) DC and have already acquired Type-1 functional polarization, based on expression of the Tbet reporter protein (Tbet-ZsGreen) *in vivo*. PNAd$^+$ HEV are not formally required for early recruitment of naïve T cells into the TME since these structures do not become discernible until later time points [i.e., day 5; Figure 1 and (5)]. B220$^+$ B cells recruited into the TME as a consequence of treatment with DC.Tbet cells are not organized into follicle-like structures during the day 2–5 time period, but may become organized in this manner at even later time points (i.e., ≥day 9 post-therapy), based on previous reports employing alternate immunotherapeutic interventions, such as ch14.18-LTα (8). While therapeutic benefits in our model were largely T cell-dependent and detectable prior to the establishment of formal TLO structures (based on the development of B cell follicles), the presence of "mature" TLO in human tumors has been associated with better clinical prognosis.

migrate to tumor-draining SLO, and the corollary requirement of SLO-primed T cells to be recruited back into tumor sites, TLO may operationally increase the efficiency of anti-tumor T cell cross-priming and the therapeutic action of such T effector cells in the TME. Translational studies clearly suggest that TLO formation in the TME may be therapeutically fostered by the directed delivery of LTβR ligands in both protein- and gene-based formats. At present, LTβR agonist-based therapies are in their infancy with only rhLTα thus far evaluated in phase I clinical trials, where minimal anti-tumor efficacy was observed in patients with melanoma or renal cell carcinoma (83). The inability to focus this potent TLO induction agent in appropriate sites of disease in order to most effectively recruit and activate protective immunity in treated patients must be considered a possible limitation to the current treatment strategy. The ligation of rhLTα to a cancer-specific antibody or the intratumoral administration of this agent could improve anti-tumor efficacy and coordinately reduce current off-target toxicities [i.e., grade III chill, grade III fever, and grade III dyspnea; Ref. (83)].

Improved targeted delivery of LTβR ligand or downstream chemokine gene therapies is conceptually attractive given pre-clinical results in murine tumor models. To date, however, only a recombinant adenovirus encoding hCCL21 has been developed for phase I clinical application, with the intent to deliver rAd.CCL21-infected patient DC directly into tumors in patients with late stage human lung cancer (84) or in vaccine formulations applied to patients with melanoma (85). Although this approach requires further optimization of the clinical vector based on low levels of CCL21 produced by engineered DC, melanoma patients treated at the lowest dose tier of DC.CCL21 did develop lymph node-like structures based on immunohistochemical analysis of vaccination site biopsies (James Mulé, personal communication). Our own pre-clinical data would support the clinical application of DC.Tbet directly into accessible tumor lesions as a means to drive TLO development and protective immunity in the TME. Motivation for the development of prospective phase I trials using DC.Tbet cells will be intensified when the underlying mechanism of action for this treatment modality has been defined.

Finally, in a related note, antagonists of LTβR ligands (such as BTLA and CD160) have been shown to be immunosuppressive molecules in inhibiting DC homeostasis as well as the protective effector functions mediated by T cells and NK cells (74, 86–90).

It is therefore conceivable that endogenous levels of TLO development and corollary anti-tumor immunity may be bolstered therapeutically as a consequence of administering agents (i.e., antagonist antibodies or DC genetically engineered to produce specific inhibitors locoregionally in the TME) that are capable of blocking the action of BTLA or CD160 *in vivo*.

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Dendritic cells in cancer immunotherapy clinical trials: are we making progress?

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Dendritic cells (DC) have been tested in cancer immunotherapy clinical trials for two decades. Over this time, the methods of DC culture (or manufacture) have evolved, the approaches for antigen loading have broadened, the maturation signals have varied and different sites of administration have been tested. The post-vaccination immunologic questions asked have also varied between trials and over time. In this review, I will consider multiple aspects of DC-based vaccines tested in cancer patients, including the cell culture, antigen loading, maturation, and delivery, as well as what we have learned from testing immune responses in vaccinated patients who have benefited clinically, and those who have not measurably benefited.

Keywords: cancer vaccines, tumor immunology, antigen presentation, dendritic cells, antigen loading

INTRODUCTION

BEGINNINGS

Dendritic cells (DC) were first identified in the early 1970s (1). However, the extremely low frequency of these cells in peripheral blood and many tissues made experimentation with DC challenging. General agreement on cell surface markers to uniquely identify "DC" from other myeloid lineage cells was another early hurdle in the field that was surmounted (DC are, at a minimum, large, granular lymphocytes that are MHC class I, MHC class II, and CD86 high, **Figure 1**). The more widespread investigation of DC identity and biology, and subsequent clinical testing of DC-based vaccines required methods for small and large-scale culture and expansion of DC progenitors *in vitro* (2). Methods were initially identified for expanding DC from human peripheral blood monocytes with granulocyte-macrophage-colony stimulating factor (GM-CSF) (3, 4) and eventually, similar approaches and surface markers were found that could be utilized for both human and murine systems. After these advances, the field was wide open.

In one of the earliest trials, Mukherji et al. (5) used intradermal injection of MHC class I-restricted MAGE-1 peptide-pulsed and GM-CSF-cultured monocytes to treat three HLA-A1$^+$ patients with advanced metastatic melanoma. They observed autologous melanoma-reactive and peptide-specific CD8$^+$ T cell responses, but no significant therapeutic responses. Such very early clinical results supported the safety and immunologic activity of these cells in cancer patients.

FIRST GENERATION CLINICAL TRIALS

The early clinical trials of DC-based cancer immunotherapy established the general safety and feasibility of this cancer vaccine strategy, and its lack of toxicity compared with other cancer treatment approaches (e.g., chemotherapy, radiation). Importantly, a small number of positive clinical responses and the clear demonstration that the goal of anti-tumor immune activation was achieved, bolstered the field, and supported additional trials. There have been several recent DC vaccine reviews published that are excellent, and that give additional details (6–8). The few early trials highlighted below are important, but small, and did not utilize standardized manufacture procedures throughout the clinical trial.

One of the first reported clinical trials that described the ability of tumor antigen-pulsed DCs to elicit a tumor-specific T cell response and yield a clinical response was published by Hsu et al. (9). In this study, four patients were treated with low-grade follicular B-cell lymphoma resistant to chemotherapy. The DCs were pulsed with target antigens of clonal immunoglobulin (idiotype) expressed by the non-Hodgkin's lymphoma, a tumor-specific, unique antigen. Patients were immunized with DC followed by booster injections of idiotype protein and keyhole limpet hemocyanin (KLH, as an immunogenic xenoantigen as well as heterologous "help" to activate CD4$^+$ T cells) as well as a final DC boost infusion given 5–6 months later. All four patients developed cellular proliferative responses specific to their own idiotype protein. More importantly, one patient had a complete tumor regression, a second patient had a partial regression, and a third patient resolved all evidence of disease. This very small study was an important proof of principal for the clinical potential of DC vaccines.

While the study performed by Mukherji et al. (above) evaluated monocyte-derived antigen presenting cells (APC), it may not have formally tested a more fully differentiated DC because the culture contained GM-CSF, but it lacked IL-4. The first clinical trial using the monocyte-derived DC that have been most commonly used in clinical trials (including both GM-CSF and IL-4 in the monocyte precursor culture) was performed by Nestle et al. (10). Sixteen melanoma patients were treated using autologous monocyte-derived DC pulsed with a cocktail of gp100, MART-1, tyrosinase, MAGE-1, or MAGE-3 peptides chosen to suit the

individual patient's class I HLA molecules. In addition, DC pulsed with autologous tumor lysate were used to treat another four patients. To provide antigen non-specific $CD4^+$ T cell-mediated help for the $CD8^+$ T cells, KLH was included during antigen pulsing. DC were injected directly into uninvolved lymph nodes. Patients received 6–10 injections of 1×10^6 cells every 1–4 weeks. Tumor regression was seen in 5 of the 16 patients, including two complete responses lasting over 15 months. Tumor regressions occurred in skin, soft tissue, lung, and pancreas indicating an impact on the clinical course of metastasizing melanoma, regardless of metastatic site.

As with many of the early trials (examples here and others), a variable number of DC vaccine administrations, consisting of different cell numbers and boost injections were delivered, and multiple types of antigen loading strategies were used. These earliest clinical studies were more proof of principle for the *in vivo* activity of DC, and less a formal testing of a specific DC vaccine approach.

In another melanoma clinical trial, Banchereau et al. (11) evaluated immune and clinical responses in 18 patients with metastatic melanoma after injecting DCs pulsed with peptides (MART-1, tyrosinase, MAGE-A3, and gp100) subcutaneously. They utilized $CD34^+$ hematopoietic progenitor cells as an alternative source of DC. DC were administered in a dose-escalation design. Enhanced antigen-specific immune responses to at least one of the peptides were seen in 16 or 18 patients, and 6 of 7 patients with immunity to two or fewer antigens had progressive disease after the study ended, while only 1 of 10 patients who responded to more than two antigens had tumor progression. This larger and more standardized study showed that broad immune responses to multiple tumor antigen-derived peptides correlated with better clinical outcome, one of the first studies showing that statistically significant correlation between immunity induced from DC vaccines and clinical outcome.

It is clear from the clinical trials described above, that most clinical trials are unique, they involve individual patient vaccination approaches and single clinical trial arms, and it is difficult to compare them. Monocytes and $CD34^+$ progenitors; complex tumor lysates containing normal, tumor-associated/shared and tumor-specific/private antigens, or synthetic MHC class I-restricted peptides; injection into blood (i.v.), skin (s.c. or i.d.), or lymph nodes (i.n.); are all parameters playing unclear roles in any clinical responses seen (**Figure 2**). The initial lessons learned were simply that DC-based vaccines were safe, feasible, and had the potential to promote clinically significant tumor regressions.

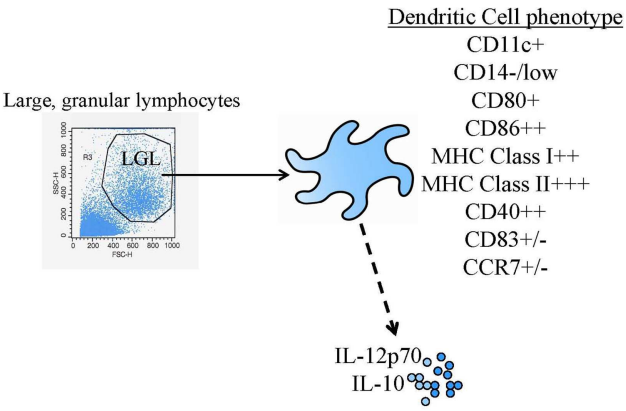

FIGURE 1 | Common DC vaccine phenotypic and functional assessments. The diagram shows the most common identity (flow cytometric phenotyping) and potency (cytokine production) tests performed.

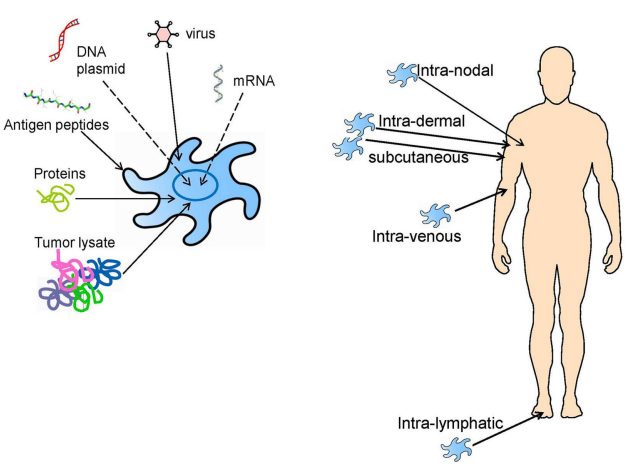

FIGURE 2 | DC vaccine loading and administration approaches that have previously been tested in clinical trials, and which are still being investigated today.

LIMITED CLINICAL RESULTS FOR DC-BASED VACCINES

In 2004, Rosenberg et al. published an article on the state of active specific immunotherapy cancer trials (12). They analyzed 9 years of data (1995–2004), essentially all of the early, or "first generation" trials. Overall, they reviewed 1,306 solid tumor patients using the modified Response Evaluation Criteria in Solid Tumors (RECIST) in which clinical response is defined as at least 50% reduction in the sum of the products of the perpendicular diameters of all lesions without 25% growth of any lesion or the appearance of new lesions. With an overall therapy-induced tumor regression rate of only 3.3% in patients vaccinated with synthetic peptides, "naked" DNA, peptide-pulsed DC, recombinant vaccinia viruses, recombinant fowlpox viruses, or recombinant adenoviruses expressing various tumor-associated antigen (TAA), the results were grim for vaccine approaches in general. Of these immunization methods, peptide-pulsed DCs seemed to be the most effective strategy, with 7.1% of treated patients exhibiting tumor regression. While this frequency of response was higher than those frequencies found for other vaccination strategies, the clinical response was still low.

DC VACCINE COMPLEXITIES

Unlike chemotherapy, immunological vaccines have not followed a linear dose-response effect. Instead, because immunologic vaccines depend on the complex interactions of a large number of variables, many of which are difficult to test: (1) the administration route (s.c., i.d., i.v., i.n., and more recently, intra-lymphatic, i.l.),

(2) minimum immunogenic dose, (3) higher dose effects, (4) vaccination schedule (weekly, monthly, or multiple times in a week or month), (5) immunological adjuvant type, and (6) the existing state of host immunological competence. There have been attempts to make "immune competence" a criterion for trial enrollment. A standardized skin test to recall antigens like tetanus and mumps was investigated, but the results were unrelated to vaccine immune response. Better measures of "immune health" are under investigation (13) but there are no clear definitions to date that might serve to identify patients who are most likely to benefit. Any alterations of these many variables can impact the patient immunologic as well as clinical outcome following therapeutic immunization.

The majority of patients treated in these earlier studies were late-stage metastatic patients that were heavily pretreated with conventional chemotherapeutic drugs prior to immunizations. Not only do late-stage tumors have potent immune-inhibitory functions well established both in the tumor microenvironment and systemically, but many traditional chemotherapies have also been shown to non-specifically decrease the number of leukocytes in recipients, making metastatic patients severely immune-compromised. Such patients would also be expected to have multiple tumor resistance mechanisms in place (e.g., infiltrated regulatory T cells, myeloid-derived suppressor cells, and other immature and skewed macrophages, immuno-inhibitory cytokines, and genetic heterogeneity in tumor subclones). Assessment of tumor infiltration and inflammation is being investigated as a biomarker for responsiveness to not only vaccines, but also other immunotherapy approaches and traditional cancer treatments (14, 15), but these areas of investigation are relatively recent and still being validated.

There are several other possibilities to explain the poor clinical response to these vaccines. The immune system, while potentially effective, is limited by the frequency of responders that can be stimulated by vaccinations. Even if TAA-specific responses were stimulated by immunization, it is possible that the bulk tumor mass was too large at the time of treatment for the available effector T cell population to infiltrate it and eliminate it efficiently. It is also possible that while the vaccine-targeted antigens are expressed by the tumors, their derivative peptides are not presenting on the cell surface in the context of MHC class I molecules, making the tumor cells effectively invisible to $CD8^+$ T cell recognition. Tumors can down-regulate antigen processing machinery molecules, including β-2-microglobulin (16, 17). Another possibility is that TAA used for vaccinations were not expressed by targeted tumors because metastatic deposits do not necessarily express the same repertoire of antigens as the primary tumor or that TAA-derived peptides used were not effective at eliciting high-avidity T cell responders. This heterogeneity has been observed in melanoma (18). The highest avidity T cells specific for self antigens may have been deleted during the development of the immune system by normal negative selection. Therefore, for some patients treated in early DC vaccine trials, instead of receiving a vaccine tailored to the individual patient's TAA repertoire, these individuals may have been treated with arguably irrelevant non-presented or weakly immunogenic antigens that led to a clinically meaningless immune response. Vaccines targeting only $CD8^+$ T cells, with short MHC class I-restricted peptides, may have only been able to activate "helpless" $CD8^+$ T cells with functional defects (19). Lastly, some tumors have evolved cell-autonomous resistance to immune-mediated killing.

SOURCES OF TUMOR ANTIGEN: IMMUNE TARGET COMPLEXITIES

Tumors are not homogenous tissues that can be effectively treated with a single antigen epitope vaccination tactic. Tumors vary in physiological location (primary tumor sites and metastatic sites), TAA repertoire, vascularization, surrounding stroma, and other properties. Some tumor types are considered more "immunogenic" due to spontaneous immune infiltration and have, therefore, been an early focus for many DC-based immunotherapy trials (melanoma, renal cell cancer). These variations in tumor biology, immune infiltration, and microenvironment biology are observed between patients, the tissues affected, and at different time points in the malignant process. For example, when considering inclusion criteria, "stage IV cancer patients" are not a homogeneous group. Whether patients with brain metastases can be included, or those with LDH levels above normal limits must be considered, as immunotherapy vaccine clinical responses can need time to evolve, and not all clinical settings are expected to allow for immune response evolution.

When considering autologous tumor-based immunization strategies, there are types of cancer that are not generally surgically removed (pancreatic cancer, hepatocellular cancer treated with ablative techniques), so the ability to load DC with autologous tumor as a source of all potential public and private TAA may not be feasible. Established cell lines are an immortal source of antigen, but may have limited antigenic overlap with a specific patient's tumor. Cell lines may express a few known, shared TAAs, however they may not express any tumor-specific and/or mutated/private antigens that the patient's tumor expresses and which may be critical to clinical outcomes. Similarly, some tumor types may have only a few characterized shared TAA with even fewer well-defined HLA-matched peptide epitopes. Importantly, since the expression of TAA is not uniform among tumor cells and metastases, it may be critical to co-administer several antigens, rather than a single one, to avoid the possibility that the sole TAA will prove non-immunogenic or that its epitopes may not be adequately presented on the tumor cell. A long term goal in the field has been to identify the "best" TAA for targeting with vaccines. The optimal TAA would be critical to survival of the tumor cells, expressed at distant metastatic sites (not downregulatable), specific to the tumor (not expressed on normal tissues), and immunogenic. Characterized TAA were ranked by a group of experts (20), but the ideal, defined, shared targets have not been identified for many tumor types, and there remains some disagreement in the field exactly what type of antigen should be targeted.

TECHNICAL ISSUES AND REMAINING QUESTIONS REGARDING DC VACCINES

MATURATION

An early lesson learned in DC vaccine development was that the DC obtained after 5–7 days of culture with GM-CSF+IL-4 were not in an optimal state for T cell activation. These DC were subsequently referred to as "immature" and potentially tolerogenic until

triggered by a pathogen and/or inflammatory signal. Such signals serve to upregulate antigen presentation and co-stimulation molecules and function, and reduce antigen uptake. Early cocktails were sometimes donor-specific and undefined (monocyte-conditioned medium), weakly stimulatory (TNF), or contained molecules which were subsequently shown to have some negative effects (PGE_2). Currently employed cocktails can incorporate specific pathogen-derived molecules, toll-like-receptor ligands (TLRs) and other "type 1" skewing agents including interferons (21). Conversely for clinical settings other than cancer, immune suppressive cocktails can be used to push DC toward their tolerizing capabilities, for autoimmune or transplantation settings. For example, anti-sense oligo co-culture of DC with anti-sense CD80, CD86, and CD40 for treatment of autoimmune diabetes has been tested, or DC culture with vitamin A for inhibition of transplant-specific immunity is being developed (22, 23). Overall, as environmental sensors, DC can be significantly modulated by instructions delivered by maturation signals and optimal signals for DC vaccines are still being developed.

DOSE AND ROUTE

Many new therapeutic drugs are tested for the effective, maximum tolerated, and toxic doses in early clinical trials. Their routes of administration are often intravenous for quick dissemination to many anatomic sites. DC vaccine development has not yet shown significant toxicity at any dose delivered (24, 25), and there have been few suggestions of minimum required dose. Doses are largely defined as the "maximally feasible dose" from a blood draw or leukapheresis procedure of a specific duration (90 min to 4 h). Regarding route of delivery, many options have been tested and questions remain, each has positive and negative aspects to consider. For intradermal delivery, too many DC in a small volume might die *in situ*. Intra-nodal delivery may deliver the DC to an optimal site, unless they are not injected into a cellular region and are injected in fat or stroma instead (26). Intravenous delivery may send cells to lungs and liver and not secondary lymphoid tissues. Not all tumors are accessible for intra-tumoral injection (which has been tested with unloaded DC to allow DC to directly sample TAA), and that environment might be harsh and result in quick loss of DC function or viability *in vivo*. Intra-lymphatic delivery may also be immunologically ideal (like intra-nodal), but is clinically challenging to administer. The optimal DC vaccine dose and route also remains to be established for human clinical trials.

SECOND GENERATION TRIALS AND LESSONS LEARNED

A new generation of clinical trials was conducted from 2004 to 2012, testing new hypotheses based on the lessons learned from the first generation, proof-of-principle studies. One key area of change has been the use of defined, optimized cytokine cocktails and pathogen-derived agonists to mature DC. The individual constituents of these cocktails have an important impact on DC biology, including the relative level of cell surface molecules (e.g., co-stimulatory molecules CD80, CD86, or maturation markers CD83 or CCR7), the amount, timing, and duration of cytokine production by DC (e.g., IL-12p70, IL-12p40, IL-10), DC lifespan, and the trafficking potential and response to chemokine gradients (21). Early, high level production of IL-12p70 may not be as optimal for T cell activation *in vivo* as delayed IL-12 production until after DC have arrived from the site of injection to the lymph node. Newer DC vaccines are not simply "mature" by a few phenotypic markers, but are treated to elicit specific types of "maturity" or immunologic skewing, based on culture conditions, and planned antigen loading and injection route strategies. Some trials testing more optimal cocktails have been performed and published results should be available soon. Other cytokine culture conditions have been tested *in vitro* [with IL-15 or IL-13 (7, 8)] but access to clinical grade reagents has been a limitation until more relaxed guidelines from the US FDA, at least for the earliest stage trials ("Guidance for Industry: CGMP for Phase I Investigational Drugs" http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070273.pdf).

Shorter duration of DC cell culture (2–3 days instead of 5–7 days with different DC maturation triggers has also been tested (27). It is difficult to identify superior DC *in vitro* given that DC differentiation may not be fixed once the DC are administered, and very few randomized trials have been performed which compare different DC *in vivo*. This also leads to one of the major limitations in DC trials. The size of most trials is quite small and hence, there are not multiple trial arms to compare experimental groups and learn the answers to important questions (with any statistical confidence). Even when trials are "negative," showing minimal positive clinical effects, there are still many variables which might explain weak results, including unavoidable patient–patient variation in generation of an autologous cellular product, and insufficient funding available to run larger, randomized trials testing specific variables, like maturation cocktails, antigen choice, antigen loading, dose, and route (28).

A blow to the field was the result of the randomized Phase III trial comparing DTIC chemotherapy for melanoma (which has a very poor efficacy record) with a matured, peptide-pulsed DC vaccine (29). The trial was stopped early due to lack of differences between the trial arms, which had similar overall clinical response rates of <6%. Many of the variables discussed above have been hypothesized to have played a part in the disappointing DC vaccine results, including DC vaccine quality and consistency in manufacture between different manufacturing sites, site of delivery (s.c. instead of i.d.), and lack of tumor-specific helper epitopes or heterologous help to promote $CD4^+$ T cell activation. With so many open questions on how best to prepare DC vaccines, there are still many possible causes for minimal clinical responses.

However, there have been a number of more successful trials published in this period. Objective clinical responses and significant immunologic responses were observed in a trial in renal cell carcinoma patients testing Muc-1 peptide+heterologous PADRE pulsed DC (s.c.) (30). These DC vaccines were combined with low dose IL-2. In another study, acute myeloid leukemia patients in remission from previous standard therapy receiving WT-1 mRNA-loaded DC vaccines showed immune activation and improved clinical outcomes (31). Another trial tested DC-tumor fusions in myeloma patients before or after autologous stem cell transplant, and observed both anti-tumor immune activation and reduction of disease (32). Interestingly, these trials all employed a DC vaccine combination strategy.

NEXT GENERATION TRIALS: WHERE ARE WE GOING?

DC FOR CANCER PREVENTION?

A new clinical setting for tumor antigen vaccination has been proposed, focused on prevention of cancer development in high risk patients without current disease (33). This vaccination setting was recently reported for a Muc-1 peptide-based vaccine in subjects with advanced colonic adenomas (but not yet colorectal cancer). The results showed that the peptide vaccine was immunogenic in 43% of the subjects and response was inversely correlated with circulating MDSC levels (34). A related Muc-1 vaccine improved survival in a murine model of colitis-associated colorectal cancer (35), supporting the further testing of preventative cancer vaccination, including utilizing DC, in a prevention setting.

ANTIGEN LOADING

For antigen loading, peptide-pulsing, transfection/transduction, and protein-pulsing continue to be used, as well as tumor lysate loading. The procedure followed for tumor lysate preparation has recently been examined. The simplest approach has been multiple rounds of freezing (in a dry ice/ethanol bath or at −80°C) and thawing (by 37° water bath). This procedure can break open cells in a manner mimicking necrosis and allow subsequent tumor protein isolation. However, there are other approaches. Tumor cell exposure to UV and gamma irradiation has been shown to mimic apoptosis, which delivers different signals to DC than necrotic cells. Recently, tumor treatment with hypochlorous acid before lysate purification was tested (36), and this method of oxidation and rapid necrosis may be superior for DC vaccine loading. Another important element may be the changes in tumor antigen expression when tumors are cultured in hypoxic conditions (5% instead of 20%) specifically mimicking *in situ* hypoxic tumor oxygen levels (37). Such improved antigen preparation approaches may yield improved clinical outcomes.

ROUTE OF DELIVERY

Based on data demonstrating that DC vaccines delivered intradermally (i.d.) show very low level (<2%) migration to lymph nodes (based most often on ^{111}In-labeling (38, 39), and that ultrasound-guided intra-nodal delivery has a risk of the vaccine being injected into fat instead of a cellular area (26), other routes of delivery have been tested. The results have varied between mice and humans, and in patients, and all tested routes of delivery have proven to be immunogenic in terms of T cell response induction. Without higher rates of objective clinical responses, identification of superior routes of delivery remain unknown. Thus far, there has also been no strict correlation between phenotypic measures, like CCR7 level on the DC surface (40), and subsequent migration. There are suggestions that the maturation cocktail used impacts migration (40) but there are no definitive answers yet. More recently, newer MRI-based DC vaccine labels have been tested (41) (D. Bartlett and P. Kalinski, personal communication, 2013) and prolonged, semi-continuous intra-lymphatic delivery of DC has been tested [(42), and P. Kalinski, personal communication, 2013]. Continued efforts at tracking DC migration *in vivo* and optimizing routes of delivery may yield more potent DC vaccines. A few such DC trials are underway.

SUPPRESSIVE DC

While the cancer and infectious disease communities have investigated optimally immune stimulating DC, the organ transplantation and autoimmunity fields have sought approaches to either maintain an "immature" DC status, or differentiate DC toward a tolerogenic or immune suppressive activity. Such strategies include pulsing DC with anti-sense oligonucleotides for co-stimulatory molecules CD40, CD80, and CD86 to downregulate these molecules (22) for prevention and treatment of diabetes, or culture with vitamin D3 and IL-10 for allograft tolerance (23). A recent Phase I clinical trial testing anti-sense CD40/80/86 oligo pulsed DC in type 1 diabetic patients showed that the cells were safe, well-tolerated, and resulted in a reduction in a subset of B cells (22). In recent preclinical primate modeling of kidney allograft survival, i.v. infusion of vitamin D3/IL-10 regulatory DC was also safe and resulted in significantly improved allograft survival (23). Further clinical development of these strategies are underway.

DC VACCINE COMBINATIONS

The field of cancer immunotherapy is now in the position of having more effective drugs encompassing not only vaccines in development, but small molecule inhibitors of key signal transduction pathways and immunologic checkpoint inhibiting antibodies. While all of these modalities, like the traditional standards of care (surgery, chemotherapy, radiation) have strengths and weaknesses, the current generation of clinical trials focuses on combinations of these approaches. DC vaccines may have limitations as stand-alone therapeutics, but in combinations they could play a role in initiating and boosting anti-tumor immunity, promoting *in vivo* cross-presentation, and promoting long term immunologic memory. Cytotoxic treatments can have multiple positive effects on the immune system, from simple release of tumor antigens as cancer cells die, to cytotoxic agent-specific effects. Release of tumor antigens allows endogenous DC to take up and present them, or for larger numbers of tumor bed injected DC to take up the broad array of released tumor antigens for T cell activation. Cytotoxic agent-specific immune effects can include: upregulation of immune stimulatory molecule expression on tumor cells (e.g., DAMPs), increased tumor antigen expression, reduced suppressor cells frequencies, as well as increased CTL proliferation and activation. The pioneering studies in this area have largely been performed in murine models, but the immune-promoting effects of non-immune-based therapeutics are now being assessed in clinical trials. Future DC vaccine combinations with rationally chosen agents may increase the effectiveness of DC vaccines (43, 44).

DC TRANSCRIPTOME ANALYSIS

An important technological breakthrough has been the ability to test the DC vaccine transcriptome. This detailed molecular characterization allows for a broader understanding of DC vaccines. Manufacturing conditions, different maturation cocktails (45, 46), and their impact on DC biology, over and above even a very thorough examination of DC surface phenotype and cytokine production (47) can be examined on a molecular basis. To date, surface expression of standard DC markers (CD80, CD83, CD86, MHC class I, MHC class II, CCR7) has not correlated significantly

with *in vivo* vaccine effects. The type-1-skewing cytokine produced by DC, IL-12p70, is actively being investigated as a potency assessment, based on its significant correlation with clinical outcome was demonstrated (48). This assay was employed after it was standardized for both spontaneous and induced expression of IL-12p70 heterodimers (47). Transcriptome analysis allows for a much broader assessment of DC vaccines, and may prove informative for predictive biomarkers of immune and/or clinical response. Such profiling has identified a type-1-skewing genetic profile expressed by DC matured with IFNγ+LPS (45), and a list of candidate genes that may be helpful for identity, stability, and potency measures of DC vaccines (46). This approach may also identify patient-to-patient variation of immunologic significance.

IMMUNOLOGIC MONITORING

Each DC vaccine clinical trial is based on the hypothesis that optimal tumor antigen presentation will promote clinically effective anti-tumor immunity. Understanding the effects of the vaccines on each patient's immune system is of utmost importance in moving the field forward. Most trials examine effector T cells activated by antigen-pulsed DC, but the cross-talk between DC and innate immune cells may also be mechanistically very important. Vaccine cell interaction with innate immune cells is expected to be variable with different types of DC cultured and loaded in different ways. DC modulation of suppressor cells, like regulatory T cells and myeloid-derived suppressor cells, may occur, and the overall immune effects may vary in magnitude and quality between peripheral blood and the tumor site. Some studies have found similar results in blood and tumor, while others have not, and studies examining DC vaccine effects at the tumor site are limited. Obtaining tumor biopsies can be challenging but well is worth the difficulties in order to understand the direct tumor site impact. Larger sized DC trials may involve multiple clinical sites, as well as vaccine manufacture sites, which necessitates careful standardization of blood processing vaccine culture and immune monitoring assay methodologies (49) as well as data reporting (50). Despite the technical challenges, careful immunologic monitoring, particularly with multiple functional assays, yields critical mechanistic insights.

CONCLUSION

Yes, we are making progress in the DC vaccine field. A more rational, defined, and data-driven approach is being employed in culturing, maturing, and antigen loading of DC (51). Fully characterizing DC vaccine transcription profiles moves far beyond the limited cell surface phenotypes previously employed. Performing more standardized trial designs where patients receive the same type of vaccine reduces variables to patient-to-patient variation instead of adding variables and may identify the most critical vaccine parameters to carry forward. More thorough, robust and standardized immune monitoring assessments are allowing to field to draw more meaningful conclusions from each trial. In the future, the next generation of optimized vaccines identified may selectively be used for individual patients, based on their tumor biology. A new generation of DC vaccine trials are underway (52) which have the potential to move this area of personalized medicine forward.

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Cancer vaccines in the world of immune suppressive monocytes ($CD14^+HLA\text{-}DR^{lo/neg}$ cells): the gateway to improved responses

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Dendritic cells are an important target in cancer immunotherapy based on their critical role in antigen presentation and response to tumor development. The capacity of dendritic cells to stimulate anti-tumor immunity has led investigators to use these cells to mediate anti-tumor responses in a number of clinical trials. However, these trials have had mixed results. The typical method for generation of *ex vivo* dendritic cells starts with the purification of $CD14^+$ cells. Our studies identified a deficiency in the ability to generate mature dendritic cell using $CD14^+$ cells from cancer patients that corresponded with an increased population of monocytes with altered surface marker expression ($CD14^+HLA\text{-}DR^{lo/neg}$). Further studies identified systemic immune suppression and increased concentrations of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes capable of inhibiting T-cell proliferation and DC maturation. Together, these findings strongly suggest that protocols aimed at immune stimulation via monocytes/dendritic cells, if optimized on normal monocytes or in systems without these suppressive monocytes, are unlikely to engender effective DC maturation *in vitro* or efficiently trigger DC maturation *in vivo*. This highlights the importance of developing optimal protocols for stimulating DCs in the context of significantly altered monocyte phenotypes often seen in cancer patients.

Keywords: $CD14^+HLA\text{-}DR^{lo/neg}$, MDSC, dendritic cells, immunotherapy, monocytes

DENDRITIC CELLS AS CANCER VACCINES

Dendritic cells are potent signal transducers in the immune system. These cells present antigen, are the essential bridge between the innate and adaptive arms of the immune system, and serve as regulators to modulate immune response to pathogenic invasion, tissue injury, and tumor development. As such, dendritic cells have received significant focus as a promising vehicle for the development of vaccines for cancer immunotherapy. We now have a more complete understanding of DC ontology with the realization that DCs exist in diverse subsets, all capable of activating T cells but possessing unique functions. DCs are classified into two broad categories. The first are monocyte-derived DCs resulting from stimulation due to inflammation or infection. The second category are steady-state DCs which include resident $CD8^+$ DCs located in the thymus, resident $CD8^-$ DCs in the spleen, plasmacytoid DCs (pDCs), migratory DCs, and Langerhans cells [reviewed in Ref. (1, 2)]. Each of these classes of DCs has been demonstrated to play a key role in immune surveillance and response but for the purpose of DC-based vaccines for immunotherapy in cancer, the focus has been on $CD14^+$ monocyte-derived DCs.

The *in vivo* pathways associated with the development of dendritic cells from monocyte precursors and the mechanisms and consequences of pathogenic activation have been described (3, 4). Briefly, DCs arise from monocyte progenitors into an immature state (iDC) responsible for immune surveillance via pathogen detection. Once activated, iDC further differentiate into mature dendritic cells (mDC) and travel to lymph nodes to activate the adaptive (typically T- and B-cell responses) and innate immune response (5). DCs also play a role in limiting the immune response against self antigen (self-tolerance) as well as limiting response to tissue damage in the absence of pathogenic signals (6). iDC can suppress immunity and have been shown to be capable of eliminating antigen-specific T cells (7). Restriction of the capacity of iDC to differentiate into mDC has been a mechanism used by viruses, parasites, and bacteria to maintain a state of self-tolerance and to enable microbial pathology (8–10). Thus, manipulation and maintenance of a state of iDC with a block on the ability to differentiate into mDC is a key mechanism of immune suppression.

To generate mDC *in vitro* for clinical use, the $CD14^+$ monocytes are the preferred precursor due to their abundance and ease of collection. $CD14^+$ monocytes are purified from mononuclear cells via adherence to plastic, antibody selection, or size centrifugation and used as source material to differentiate DC. To drive the immune response, the DCs are pulsed with tumor antigens in the form of peptides, RNA, or lysates derived from whole tumors or cell lines (11, 12). Additionally, viruses can be a potent mechanism to deliver tumor antigens (13–15). Manufacturing methods reported among clinical trials vary greatly. As a variety of methods with subtle optimizations of DC cultures have been published, there are few constants (DC activation state, tumor source, patient status, underlying disease etc.) that allow useful comparisons between the growing numbers of trials and

the underlying methods and characteristics used to generate and describe the drug (in this case DC). Often, this key aspect of drug development (optimizing and describing the purity and potency of the drug) is overlooked. However, one constant regarding the majority of the trials remains; that is the use of $CD14^+$ cells as a starting material.

MONOCYTE PRECURSORS OF DC ARE OFTEN ALTERED IN CANCER PATIENTS AND ARE IMMUNE SUPPRESSIVE

In our efforts to establish a DC vaccine protocol, we worked to optimize the maturation of DCs in cancer patients. During those studies, we discovered that in many patients, $CD14^+$-isolated monocytes were incapable of differentiation into mDC using standard DC generation protocols (16–18). This result that monocytes from cancer patients were potentially altered in their capacity to differentiate into DC, led us to search for correlative markers. We identified an increased population of monocytes with an altered surface marker expression ($CD14^+HLA\text{-}DR^{lo/neg}$) in a number of malignancies (16–20) (**Figure 1A**). This phenotype has also been reported by others in melanoma (21–23), bladder cancer (24), non-small cell lung cancer (25), and hepatocellular cancer (26, 27). Our studies in glioblastoma identified evidence of systemic immune suppression and increased concentrations of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes capable of inhibiting T-cell proliferation and DC maturation that could also be re-capitulated *in vitro* co-culture systems using tumor cell lines (18). These same immunosuppressive monocytes have been characterized with increased populations in bladder carcinoma that significantly correlate with decreased T-cell proliferation and IFN-γ production (24). These cells suppress immune function in multiple ways (**Table 1**), and therefore must be considered for any approach to DC vaccine strategies.

There is also mounting evidence that correlates increased concentrations of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes in patients with poor clinical outcome. Populations of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes and TGF-β levels were significantly expanded in metastatic melanoma patients as compared to healthy donors and correlated to a lack of response to administered granulocyte–macrophage colony-stimulation factor (GM-CSF) vaccine (22). Increased $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes correlated to both extrathoracic metastasis and poor response to chemotherapy in non-small lung cancer patients (25). Increased $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes are associated with more aggressive disease and poorer prognosis in lymphoma (16) and hepatocellular carcinoma (40). Increased $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes were associated with decreased time to progression in patients with chronic lymphocytic leukemia (CLL) (19). Increased $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes and decreased $CD4^+$ T cells can predict poor overall survival across a number of malignancies (20).

The finding that $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes are detectable systemically in patients with a variety of malignancies and that they are functionally immune suppressive raises important questions regarding their influence in *ex vivo* DC vaccine preparations. To address this, we have studied the effects of these altered monocytes on mDC generation across several cancer types using an *ex vivo* culture system. Briefly, $CD14^+$ mononuclear cells were isolated from buffy coats or apheresis leukoreduction system

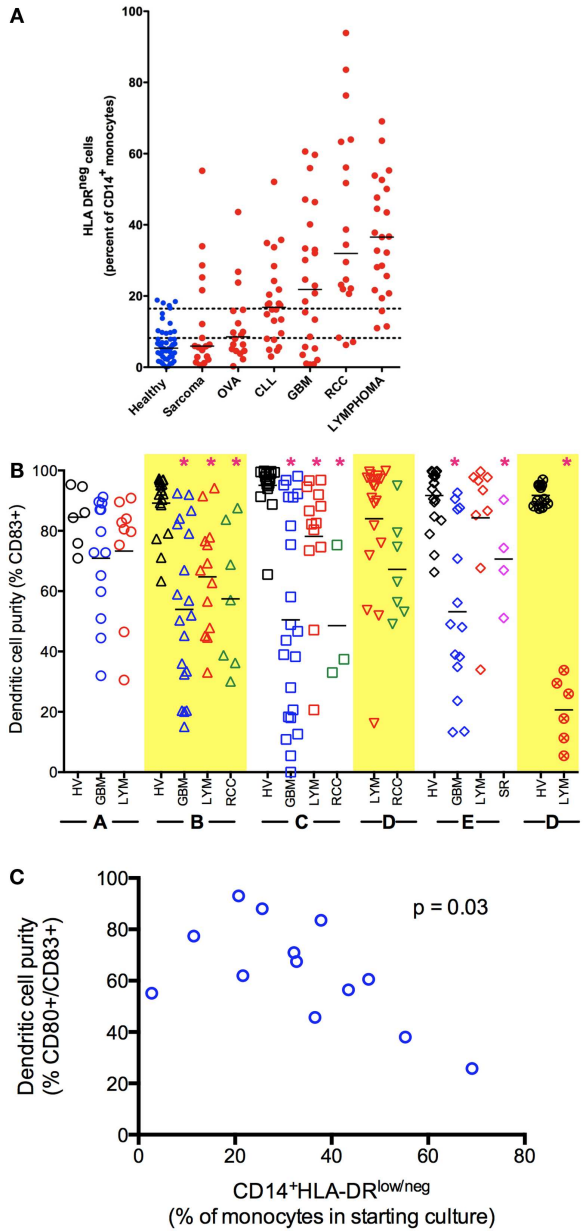

FIGURE 1 | Monocyte and dendritic cell defects in cancer. **(A)** Cancer patients have an increased percentage of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes in circulation. Peripheral blood of healthy volunteers and cancer patients was analyzed by flow cytometry for immune phenotype. **(B)** Monocytes from cancer patients have decreased capacity to differentiate to mDC ($CD83^+$) under a variety of stimulation conditions. Monocytes were selected from blood of healthy volunteers (HV) or cancer patients (GBM, glioblastoma multiforme LYM, B-cell lymphoma; RCC, renal cell carcinoma; SR, sarcoma) by $CD14^+$ immunomagnetic beads and cultured under different methods as labeled in *X* axis. Method A, fast-DC (28); B, *ex vivo* media with 5 days culture as described (29, 30); C, 5 days culture in StemLine media and GM-CSF, maturation factors TNFα and PGE2 added in the last 2 days of culture; D, method C with IL-4 added for 5 days of culture; E, method D with poly I:C added to maturation factors; F, method D with CpG used as maturation factor in place of TNF-α ($^*p < 0.05$). **(C)** Decreased generation of mDC correlates with increased percentage of $CD14^+HLA\text{-}DR^{lo/neg}$ in the monocytes selected for culture (Method B).

Table 1 | Methods of CD14+HLA-DRlo/neg immune suppression.

CD14+HLA-DRlo/neg functions	Targeted effect	Reference
Altered STAT signals	Resistance to cytokine/TLR signaling	(16, 24)
Increased IDO expression	Inhibits T-cell function	(31–33)
Increased arginase expression	Inhibits T-cell function	(12, 24, 34)
Prevention of DC maturation	Promotes immune tolerance	(16–18)
Altered co-stimulatory expression	Reduces T-cell stimulation	(12, 14, 15, 35)
Altered cytokine expression	Reduces T-cell stimulation	(15, 35)
Decreased antigen uptake	Reduces antigen-specific T-cell responses	(35)
Increased iNOS and NOX2 production	Reduces T-cell stimulation	(36)
Increased VEGF	Inhibits DC differentiation	(37)
Depletion of cytosine	Inhibit T-cell activation	(38, 39)

chambers of normal donors using immunomagnetic selection (41). Control DCs were cultured with 1% human AB serum, stimulated with GM-CSF and IL-4 (base media) for 3 days when one-third volume of fresh base media was added. Non-adherent cells were collected on day 6, re-suspended in base media with the addition of tumor necrosis factor alpha (TNF-α) and prostaglandin E2 (PGE2) to mature the DCs. This recipe is based on a classic method of generation of mDCs (28, 29, 41). Alternatively, the system was modified using a serum-free media and changes in cytokines to generate mDCs (**Figure 1B**).

Cancer patients showed significant deficits in the ability to generate mDCs independent of the underlying tumor. There is also substantially more variability in the efficiency of DC generation using monocytes from cancer patients (**Figure 1B**). While we primarily used CD83 up-regulation as indicative of DC maturation, we also noted a lack of CD80 expression and specific functional deficient of these cells. Increased efficiency of DC maturation can be correlated with decreased presence of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes in the starting culture (**Figure 1C**). However, we could consistently improve the ability to generate mDCs using serum-free methods with the addition of IL-4. Even so, it was difficult to recapitulate the efficient generation of mDCs we observed using monocytes from healthy volunteers compared to cancer patients. Knowing that $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes have significant capacity to influence *ex vivo* DC cultures implies that these cells and the pathways to both generate and eliminate them are high-value targets to improve cancer therapies. It is striking to note that these effects occurred in the complete absence of tumors and in the continual presence (for days) with the cells in excess cytokines. This deficit likely represents a significant block in the differentiation pathway. *This strongly suggests that immune stimulation in vivo, even with precise targeting of the pathways known to convert mature DC, is unlikely to efficiently trigger mDC maturation in patients.* Further understanding of the biology of these $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes is needed; strategies to overcome the effects of these cells can lead to better DC generation and immune reconstitution.

IMPLICATIONS OF $CD14^+HLA\text{-}DR^{lo/neg}$ ON DC-BASED CANCER VACCINES

Most currently active DC-based cancer immunotherapy protocols differ in either the cell source or some of the methods associated with the generation of DC. The most common approach has been the *ex vivo* generation of mature DCs from patient myeloid-derived monocyte precursors by co-culturing with GM-CSF and various cocktails of cytokines and TLR agonists to produce mature DCs. Optimizing DC culture conditions using normal healthy donors will likely not directly translate into the protocols needed for cancer patients. It will be important for those protocols that use $CD14^+$ cells to generate their DC product from primary patient samples to confirm and optimize the manufacturing method and assure that potent DCs are being generated. In our hands, a serum-free method that includes IL-4 is a good starting point. Adequate sampling size of the patient population is needed to determine the range of differentiation efficiency in each specific cancer patient population to inform the design of release criteria for vaccine manufacturing. Our data also have clear implications for other approaches attempting to mediate anti-tumor immune stimulation. *Adjuvants known to work in healthy people may not work in cancer patients if their approach is to target the DC or DC differentiation pathways.*

As we improve our understanding of the importance of $CD14^+HLA\text{-}DR^{lo/neg}$ monocytes in promoting immunosuppression, it is imperative that we adjust our clinical practices to ensure effective outcomes for patients using DC-based immunotherapy. This will require continued efforts to develop optimal protocols for generating *ex vivo* DC vaccine preparations and testing these protocols in individual patients. The complexity of the human immune system and individual tumor micro environments will likely require an element of individualized protocol development to achieve optimal clinical benefit.

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