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Prog Retin Eye Res. Author manuscript; available in PMC 2022 January 01.

Published in final edited form as:

Prog Retin Eye Res. 2021 January ; 80: 100877. doi:10.1016/j.preteyeres.2020.100877.

Plasmacytoid Dendritic Cells in the Eye

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Abstract

Plasmacytoid dendritic cells (pDCs) are a unique subpopulation of immune cells, distinct from classical dendritic cells. pDCs are generated in the bone marrow, and following development, they typically home to secondary lymphoid tissues. Nevertheless, while peripheral tissues are generally devoid of pDCs during steady state, few tissues, including the lung, kidney, vagina, and in particular ocular tissues harbor resident pDCs. pDCs were originally appreciated for their potential to produce large quantities of type I interferons in viral immunity. Subsequent studies have now unraveled their pivotal role in mediating immune responses, in particular in the induction of tolerance. In this review, we summarize our current knowledge on pDCs in ocular tissues in both mice and humans, in particular in the cornea, limbus, conjunctiva, choroid, retina, and lacrimal gland. Further, we will review our current understanding on the significance of pDCs in ameliorating inflammatory responses during herpes simplex virus keratitis, sterile inflammations, and corneal transplantation. Moreover, we describe their novel and pivotal neuroprotective role, their key function in preserving corneal angiogenic privilege, as well as their potential application, as a cell-based therapy for ocular diseases.

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Author Statement

All authors have read the final version of the manuscript and agree with the content of the manuscript. PH, AJ, and AA have filed three patent applications, which are in part related to the findings reviewed in this manuscript.

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Keywords

plasmacytoid dendritic cells; tolerance; transplantation; viral keratitis; angiogenesis; neuroprotection

1. Introduction on Plasmacytoid Dendritic Cells

1.1. Identification of Plasmacytoid Dendritic Cells

The discovery of plasmacytoid dendritic cells (pDCs) began with observations in human tissues. In 1958, the pathologists Lennert and Remmele noted a previously unappreciated cell type in human lymph nodes in cases of non-specific lymphoid hyperplasia. These cells appeared medium sized, were referred to as a “lymphoblast”, and were noted to be present in clusters (Lennert *et al.* 1958). Considering that these cell clusters were later observed in T cell-associated (paracortical) areas of lymph nodes, and that electron microscopy studies indicated an abundant rough endoplasmic reticulum resembling plasma cells (Muller-Hermelink *et al.* 1973), Lennert *et al.* later referred to them as “T-associated plasma cells” (Lennert *et al.* 1975). The advent of immunostaining techniques later revealed that these cells expressed the T-helper (Th) marker CD4 (clones Leu-3a and OKT4), but lacked common T cell and B cell lineage markers; thus, they were described as “plasmacytoid T cells” (Feller *et al.* 1983, Muller-Hermelink *et al.* 1983, Papadimitriou *et al.* 1983, Vollenweider *et al.* 1983, Harris *et al.* 1987). Yet, more extensive immunophenotyping revealed that these cells also expressed the myelomonocytic markers Ki-M6 and Ki-M7 (Horny *et al.* 1987), and thus in 1988 Facchetti *et al.* proposed renaming them to “plasmacytoid monocytes” (Facchetti *et al.* 1988). In 1997, Grouard *et al.* showed that freshly isolated plasmacytoid T cells/monocytes were morphologically nearly identical to CD4^{+} CD11c^{neg} Lin^{neg} immature cells, which differentiate into dendritic cells (DCs) in human peripheral blood, and upon cultures with interleukin (IL)-3 and CD40 ligand, they effectively promoted proliferation of naïve CD4^{+} CD45RA^{+} Th cells (Grouard *et al.* 1997). Few months later, Olweus *et al.* confirmed the phenotype of plasmacytoid T cells/monocytes and their capacity to induce naïve T cell proliferation following *ex vivo* stimulation (Olweus *et al.* 1997). In 1999, Rissoan *et al.* then designated these cells as “type 2 DC precursors (pDC2s)”, as their *ex vivo* cultures with naïve CD4^{+} T cells demonstrated that they favored production of a Th2 cytokine profile in naïve T cells, in contrast to monocytic precursors of myeloid (conventional or classical) DCs (cDCs), which promoted a Th1 response (Rissoan *et al.* 1999). However, since further studies indicated that both cDCs and pDC2s were able to interact with both Th1 and Th2 cells (Boonstra *et al.* 2003), the term “plasmacytoid dendritic cell” (pDC) was more commonly used.

Considering the importance of interferons (IFNs) in viral infections, in entirely independent line of studies, Trinchieri *et al.* showed that an unknown type of lymphocytes isolated from peripheral blood that did not belong to B or T cells, had a strong capacity to secrete IFNs (Trinchieri *et al.* 1978). In fact, the majority of IFNs in the blood were secreted by a rare subpopulation of immune cells that was initially termed natural “IFN producing cells” (IPCs) (Ronnblom *et al.* 1983). IPCs were distinct from cDCs, monocytes, natural killer (NK) cells, T cells, and B cells (Abb *et al.* 1983, Ronnblom *et al.* 1983, Perussia *et al.* 1985,

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Fitzgerald-Bocarsly *et al.* 1988, Chehimi *et al.* 1989, Feldman *et al.* 1990, Ferbas *et al.* 1994, Svensson *et al.* 1996). Further, several studies showed that IPCs co-purified with cells with a dendritic morphology, expressed major histocompatibility complex (MHC)-II, and morphologically resembled DCs based on their large size, and veiled and ruffled morphology (Fitzgerald-Bocarsly *et al.* 1988, Ferbas *et al.* 1994). Moreover, Chehimi *et al.* demonstrated that IPCs and cDCs were distinct populations, by showing that cDCs, but not IPCs were potent inducers of strong mixed lymphocyte reactions (Chehimi *et al.* 1989). Svensson *et al.* showed that IPCs could promote T cell proliferation, suggesting that IPCs resembled immature but not mature cDCs (Svensson *et al.* 1996). Thus, by the mid-1990s cumulative evidence suggested that IPCs might belong to the DC family. In the late 1990s, Siegal *et al.*, and Cella *et al.*, independently demonstrated that IPCs in fact hold the same identity as the independently identified pDCs (Cella *et al.* 1999, Siegal *et al.* 1999).

Following the discovery of pDCs in humans, investigators aimed to unravel their murine counterparts. In 2001, a few years after unifying the identity of pDCs and IPCs in humans, Nakano *et al.*, Asselin-Paturel *et al.*, and Bjorck independently recognized a subpopulation of DCs in murine lymph nodes and spleens that displayed a plasmacytoid morphology (Asselin-Paturel *et al.* 2001, Bjorck 2001, Nakano *et al.* 2001). They demonstrated that these cells have the capacity to stimulate naïve T cells and produce IFN-α when stimulated *in vitro* and *in vivo*, proposing that they are equivalent to human pDCs (Asselin-Paturel *et al.* 2001, Bjorck 2001, Nakano *et al.* 2001). Fig. 1 demonstrates scanning electron micrograph (Fig. 1A) and transmission electron micrograph (Fig. 1B) of pDCs isolated from human peripheral blood as well as pDCs in murine spleen during steady state (Fig. 1C). Upon verification of these observations by additional groups (Brawand *et al.* 2002, Martin *et al.* 2002, O'Keeffe *et al.* 2003), later studies identified pDCs in monkeys (Coates *et al.* 2003), pigs (Summerfield *et al.* 2003), rats (Hubert *et al.* 2004), and sheep (Pascale *et al.* 2008), suggesting that pDCs may be preserved during evolution.

1.2. Phenotypic Markers of Plasmacytoid Dendritic Cells

1.2.1. Plasmacytoid Dendritic Cell Markers in Human—The original discovery of human pDCs showed that pDCs do not express CD3 (T cell marker), CD20, CD22 (both expressed by B cells and plasma cells), but do express CD4, CD68, and IL-3Rα (CD123) (Lennert *et al.* 1975, Horny *et al.* 1987, Facchetti *et al.* 1988, Grouard *et al.* 1997). Later, it was shown that pDCs specifically express blood dendritic cell antigen (BDCA)-2 (CD303) (Dzionek *et al.* 2000), Ig-superfamily receptor or immunoglobulin-like transcript (ILT)-7 (Rissoan *et al.* 2002, Cao *et al.* 2006), and share expression of BDCA-4 (CD304; neuropilin-1) with other cells (Dzionek *et al.* 2000). Further, in contrast to mice, human pDCs do not express CD11c (Facchetti *et al.* 1988, Grouard *et al.* 1997, Olweus *et al.* 1997).

BDCA-2 is a type II C-type lectin, which can take up antigens and inhibit secretion of IFN-α/β and tumor necrosis factor (TNF)-α (Dzionek *et al.* 2001, Cao *et al.* 2006). Although it is deemed pDC-specific in humans, expression of BDCA-2 is down-regulated in pDCs, when cultured with IL-3 (Dzionek *et al.* 2000). ILT-7, similar to BDCA-2, is considered a human pDC-specific cell surface receptor, which can regulate secretion of IFN-α and TNF-α in stimulated pDCs (Cao *et al.* 2006, Cho *et al.* 2008). BDCA-4, a type I transmembrane

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receptor, is a member of the class 3 semaphorin subfamily (Kolodkin *et al.* 1997), a co-receptor for vascular endothelial growth factor (VEGF)-A, and is expressed by human pDCs, as well as by some other murine and human cell populations such as immune cells (Tordjman *et al.* 2002, Bruder *et al.* 2004, Delgado *et al.* 2005, de Paulis *et al.* 2006, Ghez *et al.* 2006, Bles *et al.* 2007, Lepelletier *et al.* 2007, Battaglia *et al.* 2008, Fantin *et al.* 2010, Carrer *et al.* 2012, Mendes-da-Cruz *et al.* 2014, Miyauchi *et al.* 2018), vascular endothelial cells (Herzog *et al.* 2001) and in multiple cancers (Jubb *et al.* 2012, Li *et al.* 2016, Zhu *et al.* 2018, Ma *et al.* 2019, Yang *et al.* 2019). While this molecule has broad implications in axonal guiadance and angionegensis (Bagri *et al.* 2002, Plein *et al.* 2014), in pDCs, it may be implicated in IFN-α secretion (Grage-Griebenow *et al.* 2007) and similar to cDCs, may contribute to pDC-T cell interactions (Chaudhary *et al.* 2014).

Thus, in humans, pDCs can be indentified by their expression of CD4, IL-3Rα, CD45R/B220, BDCA-2, BDCA-4, ILT-3, and IL-7, and lack of expression of T cell and B cell lineage markers, CD3 and CD19, as well as the myeloid marker, CD11b, and cDC marker, CD11c (Table 1). Among these markers, BDCA-2 and ILT-7 are considered specific pDC markers. Accurate detection of human pDCs requires a core panel consisting of CD45, BDCA-2, IL-3Rα, ILT-7, CD11c and CD11b (Barchet *et al.* 2005, Rogers *et al.* 2013, Swiecki *et al.* 2015).

1.2.2. Plasmacytoid Dendritic Cell Markers in Mice—In mice, detecting pDCs is more sophisticated as compared to humans. Murine pDCs were originally identified as $CD11b^{neg}$ $CD4^{+}$ $CD11c^{low/int}$ $CD45R/B220^{+}$ $Gr\text{-}1^{+}$ $Ly6C^{+}$cells (Asselin-Paturel *et al.* 2001, Bjorck 2001, Nakano *et al.* 2001, Brawand *et al.* 2002, Martin *et al.* 2002, O'Keeffe *et al.* 2003). However, to date, no single marker is considered sufficiently specific to uniquely distinguish pDCs during steady state and inflammation in mice. The first purported pDC marker, the 120G8 antibody, recognizes splenic and bone marrow pDCs during steady state, and allows depletion pDCs both functionally and physically (Asselin-Paturel *et al.* 2003). A year later, plasmacytoid dendritic cell antigen (PDCA)-1 was introduced as another antibody. This antibody also recognizes and depletes pDCs (Krug *et al.* 2004). Later, Blasius *et al.* generated an antibody, which recognizes pDCs and reacts with bone marrow stromal cell antigen 2 (BST-2; also, known as CD317) (Blasius *et al.* 2006). They showed that both 120G8 and PDCA-1 antibodies in fact recognize BST-2 as well (Blasius *et al.* 2006), suggesting that all three antibodies (120G8, PDCA-1 and BST-2) share a similar target. Although in mice, BST-2 is predominantly expressed on pDCs during steady state in multiple lymphoid organs, it is also expressed by other cell lines, such as embryonal carcinoma cell line P19, and is upregulated on multiple immune and non-immune cells during inflammation (Blasius *et al.* 2006, Holmgren *et al.* 2015). Further studies showed that expression of PDCA-1 is not necessarily restricted to pDCs even during steady state, as rare subpopulations of B cells and plasma cells may share expression of PDCA-1 with pDCs (Vinay *et al.* 2010, Vinay *et al.* 2012). Current studies suggest that PDCA-1, as a type II transmembrane glycoprotein, may tether viral membranes to host cell membranes and thus, prevent the release of multiple enveloped viruses from infected cells (Martin-Serrano *et al.* 2011, Hotter *et al.* 2013). Furthermore, PDCA-1 may regulate IFN production by pDCs (Swiecki *et al.* 2012) and can amplify NF-κB signaling (Cocka *et al.* 2012).

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Another potentially specific pDC marker initially designated as 440c (Blasius *et al.* 2004), recognizes a member of the Sialic acid-binding immunoglobulin-type lectin (Siglec) family of I-type lectins, called Siglec-H, suggesting that Siglec-H is selectively expressed by murine pDCs (Blasius *et al.* 2006). Later studies indicated that pDCs share expression of Siglec-H with other immune cells, including marginal zone macrophages in the spleen, medullary cord macrophages in lymph nodes, and central nervous system microglia (Zhang *et al.* 2006, Konishi *et al.* 2017). Current data suggests that Siglec-H is an endocytic receptor, which mediates antigen uptake by pDCs and may regulate IFN and cytokine production in these cells, as well as interaction of pDCs with T cells (Blasius *et al.* 2004, Blasius *et al.* 2006, Zhang *et al.* 2006, Takagi *et al.* 2011).

Gr-1 was among the first array of markers by which murine pDCs were originally identified (Asselin-Paturel *et al.* 2001, Nakano *et al.* 2001). Prior to the discovery of murine pDCs, it was acknowledged that the Gr-1 antibody strongly reacts with granulocyte marker Ly6G, but also recognizes Ly6C as another member of Ly6 complex (Fleming *et al.* 1993). Later, it was shown that murine pDCs do not express Ly6G (Asselin-Paturel *et al.* 2003), but do express Ly6C, and thus, the antibody against Gr-1 (that recognizes Ly6C), can also detect pDCs. However, pDCs have a lower affinity to binding this antibody compared to neutrophils, and are thus, stained with less intensity with Gr-1 (Shortman *et al.* 2002). Moreover, although Ly6C is highly expressed on pDCs (Asselin-Paturel *et al.* 2003), due to its well-known common expression by subsets of multiple immune and non-immune cells (Jutila *et al.* 1988), its solo application for differentiating pDCs is discouraged (Colonna *et al.* 2004).

Another important murine pDC marker is Ly49Q, a type II C-type lectin polypeptide (Rahim *et al.* 2014). Gr-1$^+$ cells expressing Ly49Q, were shown to also express CD11c and CD45R/B220, corroborating their identity as pDCs (Toyama-Sorimachi *et al.* 2005). However, in addition to pDCs, stimulated macrophages and cDCs may also express Ly49Q following treatment with IFN-α or IFN-γ (Toyama-Sorimachi *et al.* 2005). Further, in a few murine strains, all myeloid cells express low levels of this molecule (Toyama-Sorimachi *et al.* 2005). In addition to the potential role in pDC maturation (the process of up-regulation of MHC-II and antigen presentation to prime adaptive immune cells) (Toma-Hirano *et al.* 2009), through interaction with MHC-I, Ly49Q may regulate cytokine production, including IFN-α, in pDCs (Tai *et al.* 2008).

In summary, murine pDCs express PDCA-1, Siglec-H, CD11c$^{low/int}$, CD45R/B220, Ly49Q, Ly6C, and Gr-1 (Table 1). Thus, to accurately identify murine pDCs, assessment of multiple markers, such as a core panel of PDCA-1, CD45R/B220, Siglec-H, CD11b, CD11c, Ly6C, Ly49Q, CD3, and CD19 is recommended (Barchet *et al.* 2005, Jegalian *et al.* 2009, Rogers *et al.* 2013, Swiecki *et al.* 2015).

1.2.3. Plasmacytoid Dendritic Cells Repertoire of Toll-like Receptors—

As members of the innate immune system, pDCs are equipped with a specific repertoire of pattern recognition receptors. Both in humans and mice, pDCs are known to particularly express endosomal/lysosomal receptors toll-like receptor (TLR)-7 and TLR-9, which enables them to detect single stranded RNA and double stranded DNA, respectively (Kadowaki *et al.* 2001, Krug *et al.* 2001, Hornung *et al.* 2002, Edwards *et al.* 2003).

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Although traditionally pDCs were thought to express only TLR-7 and TLR-9, some reports suggests that pDCs may also express TLRs-1, −6, and −10 (Kadowaki *et al.* 2001, Krug *et al.* 2001, Hornung *et al.* 2002, Edwards *et al.* 2003, Hasan *et al.* 2005, Raieli *et al.* 2019), and may also up-regulate TLRs-2 and −4 (Hernandez *et al.* 2011, Zheng *et al.* 2012). Fig. 1D summarizes the markers and receptors expressed by pDCs in mice and humans.

1.3. Development of Plasmacytoid Dendritic Cells

The development of pDCs is defined by their remarkable plasticity. pDCs are continuously generated in the bone marrow and after terminal differentiation, they egress into the bloodstream (Sawai *et al.* 2013, Chistiakov *et al.* 2014). However, a minor subset of pre-pDCs may enter the bloodstream and differentiate into pDCs or alternatively into cDCs, depending on the tissue environment (Schlitzer *et al.* 2012). In contrast to many other immune cells that originate from either myeloid or lymphoid progenitors, at least in mice, pDCs can arise from both common lymphoid and myeloid progenitors (Wu *et al.* 2001, Karsunky *et al.* 2003, Karsunky *et al.* 2005, Rodrigues *et al.* 2018, Dress *et al.* 2019). In fact, both lineages have been shown to lead to a common peripheral pDC phenotype with the characteristic set of pDC surface markers as described above, although myeloid-derived pDCs may show a higher cytokine secretory and T cell-stimulating capacities (Yang *et al.* 2005).

1.3.1. Plasmacytoid Dendritic Cell Development in Mice—In mice, the evidence of the development of pDCs from both lineages is strongly supported by multiple immunophenotyping studies on the developing progenitors in the bone marrow, as well as recent single cell sequencing approaches on the characterizing pre-pDCs and pDCs (Rodrigues *et al.* 2018, Dress *et al.* 2019). Our current knowledge suggests that from the myeloid pathway, pDCs are derived either directly from myeloid macrophage/DC progenitors (MDPs), or via the MDP to common DC progenitor (CDP) pathway (Karsunky *et al.* 2005). In mice, CDPs are lineage marker-negative (Lin^{neg}), indicating they are not expressing mature cell lineage markers (Karsunky *et al.* 2005). In addition, CDPs are fms-like tyrosine kinase (FLT)3^{+} (also known as CD135), receptor tyrosine kinase KIT low or intermediate (c-Kit$^{low/int}$), and macrophage colony stimulating factor receptor (M-CSFR)$^{+}$. Exposure to FLT3-ligand (FLT3-L), and to a lesser degree M-CSF, induces CDPs toward pDCs (Karsunky *et al.* 2005).

pDC developmental paradigms are encoded by the cumulative effects of several key transcription factors. Among the key transcription factors in pDC development, are transcription factor 4 (TCF-4), also known as basic helix-loop-helix transcription factor or E protein (E2–2) and its protein co-factor MTG16, as well as B-cell lymphoma/leukemia 11A (BCL-11A), and interferon-regulatory factor 8 (IRF-8) (Cisse *et al.* 2008, Ghosh *et al.* 2010, Ippolito *et al.* 2014, Sichien *et al.* 2016, Grajkowska *et al.* 2017). As with most developmental paradigms, there are counteracting players involved. The DNA-binding protein inhibitor ID-2 acts by binding to and inhibiting E2–2. Therefore, if ID-2 is expressed by CDPs, pDC development is suppressed, as E2–2 is blocked from activating its downstream targets (Ghosh *et al.* 2014). When E2–2 is expressed by CDPs, it activates the interferon regulatory factors (IRF)-4 and IRF-8, two closely related transcription factors that

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have a central role in pDC development (Tamura *et al.* 2005). Studies have demonstrated that pDCs are diminished in both IRF-4 and IRF-8 knockout animals and that thus, both IRF-4 and IRF-8 are important in pDC development (Tamura *et al.* 2005). In fact, the expression of E2–2 is not only required for the initial development of pDCs from progenitors in the bone marrow, but also for the continued maintenance of the pDC phenotype in the periphery (Ghosh *et al.* 2010).

PU.1 is another key transcription factor in pDC development that regulates the expression of the *Flt3* gene and as such, PU.1 deletion blocks pDC development (Carotta *et al.* 2010). Phosphoinositide 3-kinase, a mammalian target of rapamycin (mTOR), signals downstream of FLT3 (Sathaliyawala *et al.* 2010), and inhibition of mTOR reduces pDC numbers, whereas deletion of the *Pten* gene, a negative regulator of mTOR signaling, increases pDC differentiation (Sathaliyawala *et al.* 2010). Spi-B has also been highlighted as a master regulator of the pDC fate and is required for pDC development (Sasaki *et al.* 2012). Yet, another transcription factor, X-box binding protein-1 (XBP-1), has been implicated for both differentiation and survival of pDCs. Knockout of XBP-1 results in decreased numbers of pDCs, and stimulation of XBP-$1^{-/-}$ pDCs resulted in decreased survival (Iwakoshi *et al.* 2007). Other transcription factors involved in pDC development include DNA-binding protein Ikaros, hypoxia-inducible factor 1α, growth factor independent 1, and nuclear polyadenylated RNA-binding protein 2 (Rathinam *et al.* 2005, Allman *et al.* 2006, Balzarolo *et al.* 2012, Backer *et al.* 2017). Several studies have shown that knockout mice lacking these transcription factors show reduced, if not eliminated pDCs in the examined tissues (Rathinam *et al.* 2005, Allman *et al.* 2006, Balzarolo *et al.* 2012, Backer *et al.* 2017).

Lymphoid progenitors (LPs) can also commit to the pDC lineage. pDC precursors of lymphoid origin include the lymphoid primed progenitors (LMPP), which give rise to MDPs, and the common lymphoid progenitor (CLP) (Vogt *et al.* 2009, Sathe *et al.* 2013). Although pDCs may originate from CLPs, they may develop independent of key enzymes required for B and T cell development. In this regard, it has been demonstrated that in $RAG1^{-/-}$ mice, which lack mature B and T cells (Mombaerts *et al.* 1992), and in mice with disruptive Ig H chain mu membrane exon that lack B cells in the peripheral blood (Kitamura *et al.* 1992), the density and phenotype of pDCs is comparable to wild-type mice (Nikolic *et al.* 2002). In contrast, the lymphoid cytokine IL-7 seems to play a role in pDC development as shown in IL-7 deficient mice, which contain few pDCs in the adult (Vogt *et al.* 2009). As in the myeloid pathway, lymphoid progenitors expressing FLT3, when exposed to FLT-3L, develop into pDC (Lyman *et al.* 1993, Maraskovsky *et al.* 1996).

1.3.2. Plasmacytoid Dendritic Cell Development in Humans—In humans, a new paradigm regarding pDC development has recently emerged, with noted differences from the murine model (Collin *et al.* 2018). Given the invasive nature of bone marrow biopsies, it is difficult to obtain and analyze human bone marrow of humans. The blood is easily attainable, and studies from those hematopoietic precursors have more recently been probed (Mohamedali *et al.* 2015). In the classical model of hematopoiesis, it was thought that progenitor cells divide and give rise to cells of alternative fates (e.g., CDP giving rise to pDCs and cDCs) with equal probability. However, this notion has been challenged based on available empirical data. The revised model suggests that priming occurs early on during

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hematopoiesis, which determines the ultimate fate of the cell, although the cell will transiently pass through phenotypes that were previously considered progenitors (Collin *et al.* 2018). In fact, a study by Lee *et al.* assessing clonal assays of human $CD34^+$ progenitor cells showed no human MDP or CDP progenitors (Lee *et al.* 2017). This concept has also been supported by single-cell analysis of cord blood (Karamitros *et al.* 2018). Given the lack of empirical evidence for specific progenitors, it has been proposed that there is no need to distinguish between myeloid and lymphoid lineages, as both result from an initial lymphoid-primed multipotent progenitor (Collin *et al.* 2018). This revised model can make sense of the lymphoid and myeloid features of pDCs (such as shared cell markers with both lineages), which the previous classical model fell short of.

Aside from the revised model of hematopoiesis in humans, the developmental paradigms among mice and men are quite similar, sharing many of the same core transcription factors. E2–2 is a major transcription factor in pDC development along with Spi-B (Schotte *et al.* 2004), IRF-4, IRF-8 (Sontag *et al.* 2017), and ZEB2, Zinc Finger E-Box Binding Homeobox 2, working in conjunction with MTG16 to repress ID-2 expression (Villani *et al.* 2017), which mirrors murine pDC development. In humans, a loss of E2–2 or heterozygous mutation causes Pitt–Hopkins syndrome in which mature type I IFN-secreting pDC are significantly reduced (Cisse *et al.* 2008). Further, the cytokine thrombopoietin, along with FLT3-L, can synergistically promote human pDC development (Cisse *et al.* 2008, Nagasawa *et al.* 2008, Ghosh *et al.* 2010). Overall, it has been difficult to directly examine the ontogeny of human pDCs, as it is not feasible to perform fate-mapping experiments in humans as researchers have done in the murine systems. Fig. 2 summarizes development of pDCs from bone marrow stems cells and highlights the key transcription factors involved in their development.

1.4. Tissue Distribution of Plasmacytoid Dendritic Cells

pDCs are generally considered as rare, but potent immune cells, comprising close to only 1% of immune cells in the bone marrow. Studies in mice and humans have widely confirmed that after generation in the bone marrow, pDCs enter the blood steam, where they constitute less than 1% of immune cells (Asselin-Paturel *et al.* 2003, Chowdhury *et al.* 2010, Murray *et al.* 2019). While circulating in the blood stream, during steady state they typically home to secondary lymphoid organs, including the spleen, lymph nodes, tonsils, thymus, and Peyer's patches of the gut (Grouard *et al.* 1997, Bendriss-Vermare *et al.* 2001, Nakano *et al.* 2001, Jameson *et al.* 2002, Asselin-Paturel *et al.* 2003, Castellaneta *et al.* 2004, Omatsu *et al.* 2005, Contractor *et al.* 2007, Boor *et al.* 2019). Based on the current understanding, pDCs are typically absent in peripheral tissues during steady state, with few exceptions. However, they are recruited from the blood to sites of inflammation during microbial infections, tumors, and autoimmune conditions (Nestle *et al.* 2005, Santoro *et al.* 2005, Smit *et al.* 2006, Sozzani *et al.* 2010).

Nevertheless, few peripheral tissues do host pDCs during steady state, albeit in low densities. Among these peripheral tissues, lungs were among the first tissues in which resident pDCs were noted during steady state (Donnenberg *et al.* 2003, de Heer *et al.* 2004). Attempting to assess the role of antigen presenting cells (APCs) in the prevention of immune

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responses to allergens, de Heer *et al.* showed that pDCs reside in the interalveolar interstitium, at almost twice-higher densities as resident cDCs (de Heer *et al.* 2004). Later studies confirmed the presence of resident pDCs in murine and human lungs; however, at lower densities compared with cDCs (Donnenberg *et al.* 2003, Lommatzsch *et al.* 2007, Venet *et al.* 2010, Ten Berge *et al.* 2012).

The kidney is another peripheral tissue in which resident pDCs have been reported during steady state by Coates *et al.*, who showed that murine kidneys host CD11c^{+} CD45R/B220^{+} CD8α^{neg} cells, presumably of pDC phenotype (Coates *et al.* 2004). Resident pDCs were reported in the tubulo–interstitium, and rarely within the glomeruli of normal human kidneys, although their density was less as compared to cDCs (Woltman *et al.* 2007). Assessing the role of pDCs in vaginal herpes simplex virus (HSV)-2 infection, Lund *et al.*, showed that the murine vagina also hosts resident pDCs during steady state in a density comparable to cDCs (Lund *et al.* 2006). In line with their findings, Agrawal *et al.* later showed the presence of pDCs the cervical mucosa of healthy individuals (Agrawal *et al.* 2009).

Moreover, based on currently available RNA sequencing performed on tissue samples from 95 human individuals, E2–2 is expressed in the brain, heart, fat tissue, adrenal gland, ovary, and testis, in addition to lymphoid tissues and the lung, suggesting the potential presence of pDCs in these tissues (Fagerberg *et al.* 2014). Table 2 summarizes the non-ocular tissues, including secondary lymphoid organs and peripheral tissues in which pDCs have been reported during steady state.

Of note, although detailed morphologic assessment of resident pDCs in peripheral tissues needs further investigation, current evidence suggests that the morphology of pDCs in peripheral tissues may differ from circulating pDCs in the blood stream or lymphoid organs. As depicted in Fig. 1A–C, while electron microscopy of freshly isolated pDCs from blood circulation as well as histologic staining and MPM of pDCs in lymphoid organs (Fig. 1C) indicate that they appear as spherical cells without dendritic projections (Grouard *et al.* 1997, Jegalian *et al.* 2009), histologic staining of pDCs in the peripheral tissues, such as resident pDCs in kidney and lung, suggest that they exhibit more elongated cell bodies with few stub-like projections (Masten et al. 2006, Woltman et al. 2007). Thus, pDCs may show a distinct morphology in the blood stream and lymphoid organs compared with peripheral tissues.

2. Resident Immune Cells in Ocular Tissues

The notion of ocular immune privilege has been described decades ago (Medawar 1948); however, this concept has undergone extensive modification and revision since its initial conception (Forrester 2009, Hori *et al.* 2010). While many pillars have been proposed that contribute to ocular immune privilege, from a lack or limited expression of MHC-II on resident APCs in ocular tissues (Streilein *et al.* 1979, Wang *et al.* 1987, Baudouin *et al.* 1988), to lack of lymphatics and blood vessels (Medawar 1948), and an immunosuppressive microenvironment in the ocular tissues (Streilein *et al.* 1992, Taylor *et al.* 1994, Stuart *et al.* 2005, Hori 2008, Taylor 2009), these pillars have failed to explain immune privilege in its

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entirety (Paunicka *et al.* 2015, Hamrah *et al.* 2016, Hori *et al.* 2019). Work from several groups in the field has now demonstrated that there are in fact resident immune cells present in essentially all ocular tissues (Steptoe *et al.* 1995, Butler *et al.* 1996, Gomes *et al.* 1997, Hamrah *et al.* 2002, Hamrah *et al.* 2003, Hamrah *et al.* 2003, Hamrah *et al.* 2003, Forrester *et al.* 2005, Xu *et al.* 2007). Herein, we briefly review our current understanding on resident immune cell populations in ocular tissues and discuss what is known with respect to the function of these cells, in order to provide a framework as to how pDCs complement known functions of other immune cells in reinstating homeostasis in ocular tissues. Fig. 3A illustrates the presence and distribution of resident immune cells in ocular tissues.

2.1. Cornea

Despite the traditional view that cornea is a collagenous tissue devoid of resident immune cells, it has now been demonstrated that the normal cornea is in fact home to different populations of resident immune cells, in particular innate immune cells, including APCs (Rodrigues *et al.* 1981, Vantrappen *et al.* 1985, Brissette-Storkus *et al.* 2002, Hamrah *et al.* 2002, Hamrah *et al.* 2003, Hamrah *et al.* 2003). Interestingly, resident corneal immune cells are not distributed evenly. While the majority of immune cells follow a decrease in density from the peripheral towards the central cornea, their localization throughout different corneal layers varies among subpopulations (Hamrah *et al.* 2002, Hamrah *et al.* 2003, Hamrah *et al.* 2003).

Subpopulations of cDCs are among the first populations of resident APCs that were detected in the cornea (Rodrigues *et al.* 1981, Chandler *et al.* 1985, Seto *et al.* 1987, Hamrah *et al.* 2002, Yamagami *et al.* 2005). While initial reports, studying MHC-II, suggested that cDCs, including LCs, are confined to limbus and peripheral cornea, it was later shown that MHC-II^{neg} cDCs are also located in the central corneal epithelium and stroma, with a decreasing density from periphery to center (Hamrah *et al.* 2002, Liu *et al.* 2002, Hamrah *et al.* 2003, Hamrah *et al.* 2003). Nevertheless, upon inflammatory stimuli, corneal cDCs increase and mature throughout the cornea, expressing increased levels of MHC-II and co-stimulatory molecules (Hamrah *et al.* 2003). In addition to cDCs, macrophages are shown to reside in the posterior corneal stroma during steady state in the peripheral and central cornea (Brissette-Storkus *et al.* 2002, Hamrah *et al.* 2003, Hamrah *et al.* 2003). Nakamura *et al.* demonstrated that 2 weeks following adoptive transfer of bone marrow-derived cells from eGFP-expressing mice to irradiated, syngeneic mice, eGFP^{+} cells appear in the corneal limbus and periphery, confirming the bone marrow origin of resident corneal immune cells (Nakamura *et al.* 2005). Further studies, taking advantage of transgenic CD11c$^{DTR\text{-}eGFP}$ mice that express enhanced green fluorescent protein (eGFP) under the control of CD11c promoter, confirmed the presence of eGFP^{+} cDCs in the cornea during steady state (Knickelbein *et al.* 2009). Similar to cDCs, studies on transgenic CX_3CR1^{eGFP} mice in which macrophages express eGFP, confirmed their presence in the cornea during steady state (Chinnery *et al.* 2007).

More recently, intravital multi-photon microscopy studies on corneas of CD11c$^{DTR\text{-}eGFP}$ and MHC-II^{eGFP} mice have provided *in vivo* insights into their behavior (Seyed-Razavi *et al.* 2019). These studies have shown that during acute corneal inflammation, cDCs respond to

stimuli by exhibiting less volume and more sphericity, as well as increasing their motility, highlighting the alterations in their morphology and kinetics following inflammation (Seyed-Razavi *et al.* 2019). In fact, resident corneal APCs play important roles in mediating immune responses to corneal insults, such as during infectious keratitis, dry eye disease (DED), and corneal transplantation (Liu *et al.* 2002, Buela *et al.* 2015, Hu *et al.* 2015, Hua *et al.* 2016, Ramke *et al.* 2016, Choi *et al.* 2017, Maruoka *et al.* 2018). Following corneal transplantation, corneal cDCs have been demonstrated to migrate to the mandibular draining lymph nodes (dLNs) and elicit adaptive immune responses (Liu *et al.* 2002). Further, cDCs are also vital for corneal wound healing in diabetic mice (Gao *et al.* 2016) and in corneal nerve survival in DED (Choi *et al.* 2017). In addition to antigen presentation and wound healing (Li *et al.* 2013, Bellner *et al.* 2015), corneal macrophages contribute to angiogenesis and lymphangiogenesis under pathologic conditions (Cursiefen *et al.* 2004, Maruyama *et al.* 2005, Xu *et al.* 2007, Maruyama *et al.* 2012, Kiesewetter *et al.* 2019).

Notably, resident APCs, including both cDCs and macrophages are located closely to the corneal nerves (Cruzat *et al.* 2011, Leppin *et al.* 2014, Seyed-Razavi *et al.* 2014, Paunicka *et al.* 2015, Gao *et al.* 2016, Hamrah *et al.* 2016, Hori *et al.* 2019). However, they dissociate after corneal injury, implying that neuro-immune crosstalk has a potential role in corneal health and disease (Seyed-Razavi *et al.* 2014). Employing multi-photon microscopy in double-transgenic $CD11c^{eYFP} \times Thy1^{YFP}$ mice, we have recently assessed the significance of corneal nerves on alterations in morphology and kinetics of $CD11c^{+}$ cDCs in DED. We observed that in mice with DED, cDCs are less frequently associated with nerves, and that association with nerves diminishes alterations in cDC morphology and kinetics observed in DED (Jamali *et al.* 2020).

In addition to cDCs and macrophages, subpopulations of T cells, such as γδ T cells (Li *et al.* 2007), type 2 innate lymphoid cell (ILCs) (Liu *et al.* 2017), and NK cells (Liu *et al.* 2012) have been reported in the limbus during steady state, and are involved in maintaining corneal immune privilege (Skelsey *et al.* 2001) and wound healing (Li *et al.* 2007, Li *et al.* 2011, Liu *et al.* 2012, Liu *et al.* 2017). Although the various functions of resident immune cells have been assessed in various pathological conditions and diseases, their contribution to the maintenance of homeostasis has not been demonstrated. For instance, it is not clear if/how resident immune cells may contribute to angiogenic privilege of the cornea, or how they may interact with corneal nerves to mediate corneal nerve health and function during steady state. Further, although role of various immune cells during viral keratitis has been studied, it is not yet known how much they contribute to production of type I IFNs, an important cytokine involved in antiviral immunity.

2.2. Conjunctiva

In contrast to the avascular cornea, the conjunctiva contains a variety of resident immune cells during steady state. In fact, the conjunctiva is unique among the ocular tissues as it considered as part of mucosal immune system, which is designated as conjunctiva-associated lymphoid tissue. Immune cells in the conjunctiva are either scattered in the tissue or clustered in structured aggregates resembling follicles (Fix *et al.* 1989, Hingorani *et al.* 1997). The majority of the resident immune cells in the conjunctiva reside in the substantia

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propria, however, to a lesser extent, they are also found in the epithelium (Fix *et al.* 1989, Gomes *et al.* 1997, Hingorani *et al.* 1997).

Among innate immune cells, mast cells, NK cells, cDCs, LCs, macrophages, and rarely γδ T cells and ILCs are reported in the normal conjunctiva (Allansmith *et al.* 1978, Sacks *et al.* 1986, Sacks *et al.* 1986, Soukiasian *et al.* 1992, Baddeley *et al.* 1995, Gomes *et al.* 1997, Hingorani *et al.* 1997, Knop *et al.* 2000, Ohbayashi *et al.* 2007, Yoon *et al.* 2018). While during steady state, cDCs appear to be the most common professional APCs in the conjunctiva, LCs have also been reported in the conjunctival epithelium and sub-epithelial layers (Rodrigues *et al.* 1981, Ohbayashi *et al.* 2007), with regional differences in distribution of both (Sacks *et al.* 1986). Macrophages tend to majorly populate in the substantia propria and can be rarely found among the epithelial cells, however, they are not detected among ductal cells (Gomes *et al.* 1997). Macrophages are increased in the course of experimental allergic conjunctivitis and contribute to antigen uptake and presentation (Fukushima *et al.* 2010, Ishida *et al.* 2010). Noteworthy, the density of APCs increases in the conjunctiva during aging (Bian *et al.* 2019). γδ T cells constitute a minor subpopulation of T cells in the conjunctival epithelium and substantia propria during the steady state (Soukiasian *et al.* 1992) and are shown to play a pivotal role in promoting clinical severity and eosinophilic infiltration in the conjunctiva in murine model of allergic conjunctivitis (Reyes *et al.* 2011).

In addition to innate immune cells, adaptive immune cells are present in the conjunctiva. Plasma cells are detected in the conjunctiva, in particular in the substantia propria or superficial layers of conjunctival epithelium during steady state (Allansmith *et al.* 1978, Bhan *et al.* 1982, Franklin *et al.* 1984, Vantrappen *et al.* 1985, Sacks *et al.* 1986, Hingorani *et al.* 1997, Knop *et al.* 2000, Siebelmann *et al.* 2013). Similar to resident innate immune cells, B and T cells are more frequently found in the conjunctival substantia propria rather than epithelium (Gomes *et al.* 1997). T cell population outnumber B cells in the conjunctiva (Sacks *et al.* 1986, Gomes *et al.* 1997, Hingorani *et al.* 1997). It has also been reported that the conjunctiva is endowed with a significant regulatory T cell population (Nesburn *et al.* 2007), likely contributing to the immune privileged status of the ocular surface.

It has been suggested that development of immune cells in the conjunctiva is age-dependent; resident immune cells peak in adolescence, they tend to decline through adulthood (Siebelmann *et al.* 2013). Nevertheless, similar to the cornea, the turnover of the immune cells in the conjunctiva needs further investigation.

2.3. Choroid, Iris, and Ciliary Body

Populations of APCs have also been described throughout the entirety of the uveal tract. In the iris and ciliary body, the described resident immune cell populations are predominantly macrophages, based on their expression of F4/80. Subpopulations could also be delineated by the expression of either Mac-1 or MHC-II (Williamson *et al.* 1989). Further, less frequently, resident cDCs have also been identified within these tissues. Immunohistochemistry studies has revealed distinct localization of resident macrophages and cDCs; while cDCs are present within the epithelial layers and the stroma, macrophages reside in the substantia propria (McMenamin *et al.* 1992).

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Interestingly, the turnover rate of iris-resident macrophages and cDCs differ substantially. Bone marrow transplantation into irradiated mice indicated that cDCs have a turnover rate with a half-life of 3 days, whereas macrophages had a turnover rate with a half-life of 10–12 days (Steptoe *et al.* 1996). Further, presence of $CD34^+$ progenitor immune cells in the iris has been shown, which may suggest potential in situ renewal of at least some subtype of immune cells in this tissue during steady or following inflammatory stimuli (Vrapciu *et al.* 2014). Iris-resident cDCs have been shown to induce T cell proliferation *in vitro*, whereas macrophages failed to do so (Steptoe *et al.* 1995). Later studies revealed that while resident iris macrophages could not stimulate proliferation in unprimed T cells, they were capable of promoting the proliferation of primed, antigen-specific T cells (Steptoe *et al.* 2009). This distinction between resident cDCs and macrophages can be explained in that upon antigen uptake, resident cDCs migrate to the dLNs to induce T cell responses, whereas the resident macrophages may potentiate those responses within the tissue.

In the choroid, resident cDCs appear to have an immature phenotype, as indicated by little to no expression of the co-stimulatory molecules CD80 and CD86. The lack of these molecules suggests that these cDCs are not capable of antigen presentation, but rather, points toward a role in antigen capture (McMenamin 1999). A close association between these resident immune cells and retinal pigment epithelial cells has also been described (Forrester *et al.* 1994). Choroidal cDCs are poor antigen presenters unless activated *in vitro*. Interestingly, choroidal macrophages are poor antigen presenters, even after activation, but improve the antigen presentation by cDCs when co-cultured (Forrester *et al.* 2005). More recently, choroidal resident myeloid cells have been investigated *ex vivo* by time-lapse confocal microscopy. These experiments utilized young and aged $CX_3CR1^{eGFP/+}$ mice to address if immune-vascular associations are altered during aging. These studies, highlighting close interaction of resident immune cells and choroidal vasculature and show that the density of myeloid cells increases with age (Kumar *et al.* 2014).

In the uveal track, resident immune cells, including cDCs and macrophages play an important role in mediating immune responses, in part through acting as local APCs in conditions such as autoimmune uveitis (Butler *et al.* 1996, McMenamin *et al.* 1997, Jiang *et al.* 1999), and macular degeneration (Luhmann *et al.* 2009, Cherepanoff *et al.* 2010, Bretz *et al.* 2018). Although our understanding of the role of resident immune cells in the uveal track in disease states has significantly increased, their role in maintaining immune and vasculature homeostasis during steady state remains mainly elusive.

2.4. Retina

The retina, being an extension of the central nervous system, is host to microglia. During steady state, microglia are typically detected in three layers of the retina: (1) nerve fiber layer/ganglion cell layer, (2) inner plexiform layer, and (3) outer plexiform layer (Hume *et al.* 1983, Diaz-Araya *et al.* 1995, Provis *et al.* 1995, McMenamin *et al.* 2019). However, some reports suggest that in adult retinas, they are absent from the nerve fiber layer/ganglion cell layer. Microglia are widely implicated in mediating immune responses, neurodevelopment, neuronal survival, and synaptic pruning throughout the central nervous system (Silverman *et al.* 2018). Microglia are tissue-resident long-lived cells; however,

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during injury it has been shown that bone marrow-derived cells may infiltrate into the retina and differentiate into microglia-like cells (Xu *et al.* 2007, Kaneko *et al.* 2008).

In addition to microglia, the retina hosts small fractions of other resident immune cells, such as macrophages and cDCs. Retinal perivascular macrophages are located in close association to epiretinal vessels, extending their processes around the vessels or bridging adjacent vessels (Cuff *et al.* 1996). These cells constitute a distinct population from microglia, since unlike microglia, they lack expression of Iba-1, although they share expression of F4/80 and CD11b with microglia (Mendes-Jorge *et al.* 2009). In fact, in a series of experiments, O'Koren *et al.* demonstrated that despite similarities, it is possible to distinguish microglia from macrophages within the retina via extensive phenotyping by flow cytometry (O'Koren *et al.* 2016). Interestingly, they demonstrated that microglia, at least within the retina, maintain a stable phenotype even during neuroinflammation in the light-induced retinal degeneration model (O'Koren *et al.* 2016). More recently, it has been demonstrated microglia residing in inner plexiform and outer plexiform layers harbor distinct properties, since only survival of the microglia in the inner plexiform layer is dependent on ganglion cells' secretion of IL-34 (O'Koren *et al.* 2019). Further, solely microglia in the inner plexiform layer contribute to feedback regulation of cone-bipolar cell axons and thus, visual information (O'Koren *et al.* 2019).

During retinal injury, both retinal microglia and infiltrating macrophages contribute to removal of debris. Additionally, infiltrating macrophages re-enter the circulation, a possible indication of their antigen-presenting potential (Joly *et al.* 2009). Further, microglia have been shown to interact with retinal pigmented epithelium (RPE) cells. When injected into the subretinal space of naïve mice, microglia caused alterations in RPE cells including increased expression of pro-inflammatory and pro-angiogenic molecules, with a concurrent increase in the extent of choroidal neovascularization (Ma *et al.* 2009). It has since been demonstrated that modulating interferon-β signaling can provide benefit in choroidal neovascularization, as mice benefit from systemic interferon-β therapy in a laser burn model (Luckoff *et al.* 2016).

In addition to microglia and peri-vascular macrophages, resident cDCs have also been reported in the retina (Gregerson *et al.* 2003, Xu *et al.* 2007). In a study, using CD11c$^{DTR\text{-}GFP}$ mice, it has been shown that GFP^{+} cDCs were observed in the retina, expressing CD11b and intermediate levels of CD45, further confirming the presence of resident cDCs in the retina (Lehmann *et al.* 2010). Resident retinal cDCs selectively up-regulate MHC-II expression following retinal injury, suggesting their role in antigen presentation and identification in conditions such as autoimmune uveoretinitis (Xu *et al.* 2007, Lehmann *et al.* 2010). Nevertheless, considering the recent discovery of resident cDCs, their functions during steady state and disease needs to be further elucidated.

2.5. Lacrimal Gland

The lacrimal gland forms part of the lacrimal functional unit, key for maintenance and homeostasis of the tear film at the ocular surface. As such, resident immune cell populations within the lacrimal gland will briefly be discussed. A variety of immune cells are present in the lacrimal gland, including macrophages, cDCs, and unique subpopulations of

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lymphocytes (Pappo *et al.* 1988, Wieczorek *et al.* 1988, Gomes *et al.* 1997, Saitoh-Inagawa *et al.* 2000). In the lacrimal gland, APCs are the predominant immune cell type followed by lymphocytes.

These immune cell populations appear to be involved in the homeostatic function of the lacrimal gland. For instance, following exposure to environmental antigens or ocular immunization with toxins, such as cholera toxin, the density of antibody-secreting cells in the lacrimal gland increases, leading to an increase in tear antibody levels against the antigens (Allansmith *et al.* 1987, Saitoh-Inagawa *et al.* 2000). Also, if the parasympathetic innervation to the lacrimal gland is severed, mRNA levels of mediators such as NFκB, MHC-II, macrophage metalloelastase, and CD53 are increased at early and late time points (Nguyen *et al.* 2006). Similarly, inflammatory cytokines, such as CCL2, CCL4, IL-6, MHC-II, are increased the lacrimal glands of rabbits which were housed in warmer temperatures and/or lower humidity (Mircheff *et al.* 2011). Additionally, *ex vivo* studies have demonstrated that inflammatory cytokines such as IL-1β and IL-6 directly impact secretory function and decrease chloride flux in response to treatment with carbachol, a muscarinic agonist (Selvam *et al.* 2013). Thus, within the lacrimal gland there are complex interactions between the parasympathetic nerves, resident immune cells, and even environmental cues, likely relayed by corneal afferents (Stern *et al.* 2004). Perturbation of any of these components can result in lacrimal gland dysfunction. Additional work is warranted in this area to unravel the complexities of such interactions and to uncover contributions of various immune cells.

2.6. Perspective

Previous studies have elucidated the presence of a variety of immune cells in ocular tissues during steady state and their role in different pathological settings. This has led to the potential for therapeutic targeting of immune pathways in ocular diseases. However, our understanding of the homeostatic function of immune cells in ocular tissues is lacking. An additional area that requires further investigation are whether these immune cell populations are replenished by in situ proliferation, local progenitors, or bone marrow-derived immune cells recruited from blood stream. While it appears as though there is a distinction in functions of cDCs and macrophages in these settings, studies have indicated close associations between resident immune cells and nerves as well as resident immune cells and vasculature. Understanding the neuro-immune and immune-vascular crosstalks in these tissues remain to be elucidated.

3. Distribution of Plasmacytoid Dendritic Cells in Ocular Tissues

Considering that innate immune cells are found in ocular tissues during steady state, more recent studies have assessed if murine or human ocular tissues also host resident pDCs. Notably, considering the difficulty of accessing healthy human ocular tissues, most of our knowledge on tissue-resident pDCs is derived from murine studies. Under this section, we briefly review our current understanding on presence of resident pDCs in ocular tissues and the alterations in their density or phenotype in various ocular diseases. Fig. 3B summarizes our current knowledge on distribution of resident pDCs in the ocular tissues.

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3.1. Cornea

Investigations into the potential existence of pDCs in ocular tissues date back to 2005, when Sosnova *et al.* first showed that a subpopulation of double-positive CD45R/B220$^+$ Gr-1$^+$ cells among CD45$^+$ bone marrow-derived cells in the cornea (Sosnova *et al.* 2005). Although this study first brought up the potential presence of pDCs in normal murine corneas, considering that the expression of CD45R/B220 and Gr-1 is not restricted to pDCs, these data remained inconclusive. Extensive studies on the identification of pDCs in ocular tissues awaited until 2010, when it was demonstrated that during steady state, pDCs, initially specified as CD45$^+$ PDCA-1$^+$ CD45R/B220$^+$ cells, reside in the anterior stroma in both the central and peripheral murine cornea, with their particular localization immediately below to the corneal basal epithelium (Zheng *et al.* 2010). These studies further showed that corneal pDCs express TLR-7 and TLR-9 (Zheng *et al.* 2010), consistent with prior studies in other tissues (Kadowaki *et al.* 2001, Krug *et al.* 2001, Hornung *et al.* 2002, Edwards *et al.* 2003). In addition to CD45R/B220, co-expression of CD11c was shown on CD45$^+$ PDCA-1$^+$ cells. Interestingly, as illustrated in Fig. 4A, both populations of CD45$^+$ PDCA-1^{neg} CD11c^{high} cDCs (Fig. 4A, arrow heads) and CD45$^+$ PDCA-1$^+$ CD11c^{low} presumable pDCs (Fig. 4A, arrows) are detectable in the murine limbus during the steady state. Nevertheless, clear characterization of pDCs via confocal microscopy seems technically impractical since it requires co-staining with multiple markers such as PDCA-1, Siglec-H, CD11c, CD45R/B220, and Ly6C. Thus, unequivocal confirmation of the presence of pDCs in the murine cornea during steady state awaited thorough flow cytometric evaluations, which demonstrated that the majority of CD45$^+$ PDCA-1$^+$ CD45R/B220$^+$ cells in the cornea co-express CD11c (low), Ly6C, Gr-1, and Ly49Q, but are negative for CD11b, F4/80, Ly6G, CD3, and CD19 (manuscript under review). Interestingly, pDCs appear in the cornea during the embryonic stage of life, suggesting their early homing to ocular tissues during development (Abou-Slaybi *et al.* 2019). Surprisingly, despite their rarity in the peripheral blood and secondary lymphoid organs, pDCs constitute approximately 0.4% of total corneal cells and 15–25% of corneal immune cells (CD45$^+$ cells) in corneal single cell suspensions during the steady state. In addition studies on corneal inflammation have shown that both sterile and infectious inflammatory stimuli, including thermal cautery, stromal suture placement, or HSV-1 keratitis, result in increased corneal pDC density in both the peripheral and central cornea (Zheng *et al.* 2010, Blanco *et al.* 2017) (manuscript currently under review).

The advent of intravital multiphoton microscopy has enabled *in vivo* imaging of immune cells of interest with high resolution over time in living animals, in particular in the cornea (Sumen *et al.* 2004, Seyed-Razavi *et al.* 2019). In this regard, DPE-GFP×RAG1$^{-/-}$ transgenic mice provide a potent source for studying pDCs *in vivo*, since in these mice, which lack RAG1, GFP is expressed under the control of CD4, leaving pDCs as the solely GFP-tagged cells in the tested organs (Iparraguirre *et al.* 2008). Taking advantage of this technology and availability of the transgenic mice, precise localization, morphology, and kinetics of pDCs in the cornea have recently been studied (Blanco *et al.* 2017). The findings of the study confirmed the presence of corneal pDCs during steady state without the need for application of immunofluorescence staining, confirming their higher density in the peripheral cornea. pDCs generally appear with a central cell body and few stub-like

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extensions (Fig. 4B, arrows), however, they do not possess thin dendrites as observed in cDCs. In addition, a minor population of pDCs in the cornea harbored a round cell body without cytoplasmic extensions (Fig. 4B, arrow head). In contrast, cDCs tend to have a round cell body with multiple fine dendrites, while macrophages generally exhibit shorter stellates compared to pDCs. Further, the study showed that morphology and migratory properties of pDCs are altered during inflammation, regardless of the etiology. In fact, while pDCs are sessile during steady state, during inflammation, their migratory behavior is significantly altered, as shown by their higher mean speed and longer displacement in the cornea (Blanco *et al.* 2017) (manuscript under review). These findings suggest that corneal pDCs sense and respond to inflammatory stimuli.

Of note, in the limbus, the distribution of pDCs is uniquely organized. In DPE-GFP×RAG1$^{-/-}$ mice, GFP^{+} pDCs engulf the limbal vessels (Fig. 4C and Supplementary Video 1), and in rare occasion are found in the lumen of the vessels, patrolling the intravascular space (Jamali *et al.* 2020). In the limbus, the majority (approximately 85%) of the resident pDCs accompany limbal vessels, with higher frequency around larger vessels (Jamali *et al.* 2020). Considering the critical localization of pDCs, it might be postulated that they may promptly participate in dampening immune responses, by traveling to dLNs in order to prevent unnecessary immune responses. Further, as reviewed in the relevant sections below, they may contribute to vascular integrity and corneal angiogenic privilege.

Following identification of pDCs in murine corneas during steady state, the presence of resident pDCs in human corneas has also been confirmed (manuscript under review). Performing flow cytometry on single cell suspensions of eyebank corneas from healthy individuals, it was shown that similar to mice, approximately 1–2% of corneal single cell population express CD45, among which about 15–20% co-express BDCA-2 and BDCA-4, suggesting that the normal human cornea is also endowed with resident pDCs (manuscript under review). In a recent study on human cadaveric corneas and limbal explant cultures, Luznik *et al.* suggested the presence of pDCs as judged by expression of BDCA-2, CD123, and lack of expression of CD11c on a fraction of CD45^{+} immune cells (Luznik *et al.* 2019).. Nevertheless, further evidence is needed to support the findings of this study due to the technical shortcoming of the study, such as lack of presentation of a viability marker, fluorescence minus one controls, as well as discrepancies in presented gating strategies and the density of pDCs in the peripheral cornea.

3.2. Conjunctiva

In the conjunctiva, in an initial study in 2007, investigators reported that PDCA-1^{+} CD11c^{+} cells, as presumable pDCs, are very rarely detected in the conjunctiva during steady state. However, following allergen stimulation (without subsequent challenge), pDCs are well noticed in the subepithelial layer of the conjunctiva throughout the substantia propria of bulbar, forniceal, and tarsal conjunctiva (Ohbayashi *et al.* 2007). Further, as early as 24 hours (h) following allergen challenge, pDCs are significantly increased compared with stimulated but not challenged mice in the conjunctiva, in particular in the forniceal conjunctiva (Ohbayashi *et al.* 2007). Interestingly, pDCs reach higher densities compared cDCs (Ohbayashi *et al.* 2007). Similarly, Stern *et al.* demonstrated that while during steady

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state PDCA-1^+ CD11c^{low} pDCs constitute a minor fraction of immune cells on the ocular surface (combining corneal and conjunctival tissue), their density remarkably increases as early as 1 day following induction of DED by subcutaneous administration of scopolamine and environmental desiccating stress (Stern *et al.* 2012). Despite these initial findings that demonstrated resident PDCA-1^+ CD11$c^{+/low}$ cells in the conjunctiva during steady state and their increase following allergic or desiccating stimuli, conclusive evidence could not be drawn regarding presence of resident pDCs in the conjunctiva as only two pDC markers that can also be found on other cells, were used in these studies.

Recently, following the observation of $CD45^+$ PDCA-1^+ CD11c^{low} cells in the bulbar conjunctiva and limbus of naïve mice by confocal microscopy, the presence of PDCA-1^+ $Ly6C^+$ CD11b^{neg} F4/80^{neg} pDCs among $CD45^+$ cells in the bulbar conjunctiva was confirmed during steady state by flow cytometry. In fact, these cells constitute approximately 15% of total immune cells in this tissue. Further, immunophenotyping of these cells using fluorescence minus one controls revealed that as expected for pDCs, $CD45^+$ PDCA-1^+ $Ly6C^+$ CD11b^{neg} F4/80^{neg} cells are also CD11c^+ and Ly49Q^+, but lack expression of CD3, CD19, and Ly6G (Jamali *et al.* 2020). As depicted in Fig. 4C, the presence of resident pDCs in the conjunctiva has also been observed in DPE-GFP×RAG$1^{-/-}$ mice. Importantly, the GFP^+ cells in the conjunctiva of these mice express CD45 (Fig. 4D) as well as PDCA-1 (Fig. 4E), but lack CD3 and CD19 (Fig. 4E), confirming their identity as pDCs. Notably, although the majority of GFP^+ cells in the conjunctiva of these transgenic mice aligned with pDC identity, considering that CD4 might be expressed by other APCs, such as minor subtypes of macrophages or cDCs (Vremec et al. 2000, Bialecki et al. 2011, Abtin et al. 2014, Bain et al. 2018), together with the presence of a minor subpopulation of GFP^+ cells that lacked expression of PDCA-1, it may be postulated that a minor population of GFP^+ cells in the conjunctiva of these transgenic mice may represent subtypes of cDCs or macrophages. In this regard, although expression of other immune cell markers such as myeloid cell marker, CD11b, as well as monocyte/macrophage markers, F4/80 and CD68, was not assessed on conjunctival GFP^+ cells to further support the identity of these cells as pDCs in the conjunctiva of DPE-GFP×RAG$1^{-/-}$ mice, in the cornea they mainly appeared negative for CD68 (manuscript under review). Further, during steady state, conjunctival pDCs express moderate levels of MHC-II, higher levels of co-inhibitory molecules PD-L1 and B7-H3, and minor to negligible levels of ICAM-1, CD40, and CD86, suggesting their potential tolerogenic functions (Jamali *et al.* 2020). Similar to these findings on the phenotype of resident conjunctival pDCs, murine resident lung pDCs express negligible levels of the co-stimulatory molecule CD40, and low levels of CD80 and CD86, yet considerable levels of PD-L1 (de Heer *et al.* 2004). Further, in human lung specimens, pDCs also express negligible levels of co-stimulatory molecules CD40 and CD80, and low levels of CD86 and ICAM-1 (Demedts *et al.* 2005). Similarly, pDCs detected in the kidney during steady state do not express CD40, and only express minor levels of CD80 and CD86 (Coates *et al.* 2004).

In humans, the presence of pDCs in the normal conjunctiva has not yet been explored. However, pDCs have been detected in peri- and intra-granuloma infiltrates in conjunctival biopsies of children with primary chronic blepharitis leading to granulomatous conjunctivitis (BAïZ *et al.* 2012). In summary, current evidence indicates that pDCs reside in the

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conjunctiva during steady state, at least in mice. However, considering our limited knowledge on their life cycle, it is interesting to study the longevity of these tissue-resident pDCs and to assess how these cells keep their niche in the conjunctiva during steady state.

3.3. Choroid and Retina

Currently, there are few studies on the presence of tissue resident pDCs in murine or human choroid and retina. In a recent preliminary report, Baban *et al.* assessed the presence of $CD11c^{+}$ $CD45R/B220^{+}$ cells in human eyebank retinas by immunohistochemistry (Baban *et al.* 2015). They observed that presumable pDCs reside in the normal human retina and that pDC density is decreased in the retinas from diabetic patients (Baban *et al.* 2015). More recently, murine retinas and choroid have been shown to host pDCs during steady state (Gupta *et al.* 2017). Using flow cytometry of collagen-digested tissues, $CD45^{+}$ $PDCA\text{-}1^{+}$ $CD45R/B220^{+}$ pDCs were shown to constitute less than 5–10% of $CD45^{+}$ cells in the retina and choroid during steady state. The pDC identity of these cells has been further confirmed as they expressed CD11c and Gr-1, and are negative for CD3 and CD19 (Gupta *et al.* 2017). Using transgenic DPE-GFP×$RAG1^{-/-}$ mice with GFP-tagged pDCs, the presence of GFP^{+} cells in the choroid/retinal tissues has been demonstrated during steady state, in close proximity to the vasculature. Further, GFP^{+} cells in the aforementioned tissues expressed TLR-7 and TLR-9, the main intracellular receptors of pDCs (Gupta *et al.* 2017) (manuscript under preparation). Similar to the other ocular tissues, our knowledge is currently limited on how pDCs renew in the choroid and retina. Therefore, further studies are necessary to unravel the life cycle of pDCs in these tissues and to demonstrate how these cells regenerate following cell death.

3.4. Lacrimal Gland

As the main source of the tear aqueous layer, the lacrimal gland plays a key role in ocular surface homeostasis. Considering the putative role of pDCs in Sjögren's syndrome and DED, as well as prior research indicating the presence of immune cells, and more specifically APCs in the lacrimal gland, the presence of pDCs in the lacrimal gland has been assessed. Using multiple pDC markers, it has been shown that during steady state, approximately 3–4% of $CD45^{+}$ cells in the murine lacrimal gland are $CD11b^{neg}$ $F4/80^{neg}$ $CD3^{neg}$ $CD19^{neg}$ $PDCA\text{-}1^{+}$ $Gr\text{-}1^{+}$ $CD11c^{low}$, suggestive of the presence of pDCs in the lacrimal gland (manuscript under preparation). GFP^{+} cells were also detected in the lacrimal gland of transgenic DPE-GFP×$RAG1^{-/-}$ mice (Fig. 4F), with the majority expressing CD45 (Fig. 4G), PDCA-1, moderate to low levels of CD11c, and Gr-1 (Fig. 4H), but as expected, were negative for CD11b, CD3 and CD19 (Fig. 4H), confirming the majority of them align with a pDC identity. Of note, a minor population of GFP^{+} cells did not express PDCA-1 and were positive for CD11b and/or high expressed levels of CD11c, in the lacrimal gland, suggesting that a minority of the GFP^{+} cells may represent other immune cells, such as cDCs or macrophages (manuscript under preparation). The GFP^{+} cells in the lacrimal gland of these transgenic mice express high levels of E2–2, TLR-7, and TLR-9, further suggesting their identity as pDCs (manuscript under preparation). Taken together, these observations suggest that similar to the cornea, conjunctiva, choroid, and retina, pDCs may also reside in the lacrimal gland during steady state in mice, although in sparse numbers. Fig. 3B summarizes our current knowledge on distribution of resident pDCs in the ocular tissues.

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4. Plasmacytoid Dendritic Cell Functions

4.1. General Immune Functions of Plasmacytoid Dendritic Cells

As members of innate immunity, pDCs contribute to a wide range of functions. Despite their diverse role, they exert their functions through two main approaches: (1) secretion of soluble molecules, and (2) interaction with other immune cells. Although pDCs were originally appreciated for their production of type I IFNs, they secret multiple immunomodulatory and pro-inflammatory cytokines and chemokines (*chemo*tactic cyto*kines*), including type I IFNs (IFN-α, IFN-β, IFN-ω, and IFN-τ), type II IFN (IFN-γ), type III IFNs (including IFN-λ1 [IL-29], IFN-λ2 [IL-28a], and IFN-λ3 [IL-28b]), TNF-α, IL-4, IL-6, IL-8, IL-10, IL-12, CCL3, CCL4, and CXCL10 (Coccia *et al.* 2004, Cox *et al.* 2005, Kamogawa-Schifter *et al.* 2005, Omatsu *et al.* 2005, Ito *et al.* 2006, Decalf *et al.* 2007, Smolen *et al.* 2014, Doyle *et al.* 2019). Through secretion of these cytokines and chemokines, pDCs communicate with other immune cells and surrounding cells in tissues, in order to direct pro-inflammatory or anti-inflammatory responses.

In addition to employing their secretary machinery, pDCs regulate immune response by directly interacting with other cells of the immune system. Freshly isolated splenic pDCs, as immature APCs, display a poor capacity in inducing naïve T cell proliferation. However, following stimulation, they up-regulate expression of T cell co-stimulatory molecules, such CD40, CD80, CD86, adhesion molecule CD54, and the maturation marker MHC-II, and can promote T cell proliferation (Grouard *et al.* 1997, Nakano *et al.* 2001), albeit, with a lower efficiency compared to cDCs (Abe *et al.* 2005, Tokita *et al.* 2008). In addition to priming effector T cells, pDCs may mediate the generation of regulatory T cells (Tregs), which can suppress allospecific responses (Gilliet *et al.* 2002, Moseman *et al.* 2004, Ito *et al.* 2007).

Through these mechanisms pDCs bridge innate and adaptive immunity. Thus, it is not surprising that pDCs play a key role in the development or progress of miscellaneous conditions. In the sections below, we describe how pDCs are implicated in the pathogenesis/ immune response to pathogens, autoimmune diseases, as well as tumors, and organ transplantation in non-ocular and ocular tissues.

4.2. Role of Plasmacytoid Dendritic Cells in Infectious Diseases

4.2.1. Viral Infections

4.2.1.1. Non-ocular Viral Infections: In 1957, Isaacs and Lindenmann found that supernatants of virally infected cells produce proteins that interfere with viral replication, called interferons (Isaacs *et al.* 1957). About four decades later, investigators discovered that pDCs are the main producers of type I IFNs among immune cells upon viral exposure or following exposure to unmethylated CpG-DNA sequences typically found in viruses and bacteria (Cella *et al.* 1999, Siegal *et al.* 1999, Kadowaki *et al.* 2001). Over several years, multiple additional studies revealed that pDCs are involved in anti-viral immunity against multiple viruses (Swiecki *et al.* 2010, Swiecki *et al.* 2015). Following viral encounter, pDCs are redistributed from the circulation to the lymph nodes or peripheral tissues to the site of infection, where they secrete type I IFNs (Donaghy *et al.* 2001, Penna *et al.* 2001, Barron *et al.* 2003, Yoneyama *et al.* 2005, Gerlini *et al.* 2006, Lund *et al.* 2006, Smit *et al.* 2006,

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GeurtsvanKessel *et al.* 2008, Brown *et al.* 2009, Donaghy *et al.* 2009, Gao *et al.* 2009, Kim *et al.* 2009, Lukens *et al.* 2009, Wolf *et al.* 2009, Huch *et al.* 2010, Lehmann *et al.* 2010, Davidson *et al.* 2011, Dunmire *et al.* 2015).

Although pDCs produce type I IFNs during viral infections, particularly in the early time points after viral exposure (Krug *et al.* 2004, Smit *et al.* 2006, Swiecki *et al.* 2010, Swiecki *et al.* 2013), their contribution in eliciting immune responses and promoting viral clearance is not always imperative and dependent on the secretion of type I IFNs and pro-inflammatory cytokines and chemokines (Krug *et al.* 2002, Penna *et al.* 2002, Jego *et al.* 2003, Swiecki *et al.* 2013). In fact, the contribution of pDCs to viral immune responses goes beyond secretion of type I IFNs, as they regulate different subpopulations of immune cells, including T cells, B cells, cDCs, and NK cells (Penna *et al.* 2002, Jego *et al.* 2003, Krug *et al.* 2004, Yoneyama *et al.* 2005, Tsuchida *et al.* 2012, Swiecki *et al.* 2013, Lynch *et al.* 2018). For instance, pDC depletion in local vaginal HSV-2 infection does not affect IFN-α levels, viral load, or mortality, but absence of pDCs in systemic HSV-1 and HSV-2 infections leads to decreased IFN-α levels, as well as decreased NK cell activation and reduced production of IFN-γ by virus-specific $CD8^+$ T cells, without affecting their proliferative capacity or accumulation in the site of inflammation (Swiecki *et al.* 2013). In subcutaneous HSV-1 infection, pDCs poorly induce virus-specific T cell responses; however, in their absence, cDCs lose their capacity to prime $CD4^+$ or $CD8^+$ T cells (Yoneyama *et al.* 2005). In summary, during systemic HSV-1 and HSV-2 infections, pDCs act in concert with other immune cells including cDCs, NK cells, and T cells to direct the immune response. However, they are not indispensable in local infections.

In influenza virus infections, depletion of pDCs does not affect viral clearance and generation of virus-specific $CD8^+$ cytotoxic or memory T cells (GeurtsvanKessel *et al.* 2008, Wolf *et al.* 2009), and has controversial effects on production of anti-viral neutralizing antibodies (GeurtsvanKessel *et al.* 2008, Wolf *et al.* 2009). Further, although pDC depletion may not affect viral clearance (GeurtsvanKessel *et al.* 2008, Wolf *et al.* 2009), in the absence of pDCs, infiltration of T cells to the lungs is delayed (Wolf *et al.* 2009), which can be explained by expression of both macrophage inflammatory protein (MIP)-1α and MIP-1β chemoattractant proteins necessary for recruitment of effector Th1 and $CD8^+$ T cells (Bonecchi *et al.* 1998, Loetscher *et al.* 1998, Penna *et al.* 2002, Castellino *et al.* 2006). pDCs may even destroy viral-specific $CD8^+$ T cells through FasL-Fas signaling, when encountering lethal, but not sublethal doses of influenza virus, contributing to the mortality due to lethal infections in mice (Langlois *et al.* 2010).

Furthermore, it has been shown that in respiratoty synsytial virus (RSV) infections, pDC depletion leads to increased airway hyperreactivity, accumulation of inflammatory cells, and prevention of viral clearance (Smit *et al.* 2006). Noteworthy, the increased immunopathologic severity of the RSV infection following pDC depletion, is not only due to decreased production of IFN-α and delayed viral clearance, but also due to lack of modulation of T cell responses in the absence of pDCs, since pDC depletion enhances production of both Th1 and Th2 cytokines in the lungs and dLNs (Smit *et al.* 2006). In line with these findings, it has been demonstrated that pDC depletion augments the severity of airway inflammation induced by the pneumonia virus of mice (PVM) in neonatal mice and

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is accompanied by decreased neuropilin-1$^+$ Tregs, as pDCs express semaphorin 4A and promote the generation of neuropilin-1$^+$ Tregs (Lynch *et al.* 2018). In contrast, adoptive transfer of pDCs decreases airway inflammation through inhibition of Th1 and Th2 responses, favoring Treg generation (Tsuchida *et al.* 2012).

In murine cytomegalovirus (CMV) infection, depletion of pDCs leads to reduced levels of type I IFN and increased levels of IL-12p70, with reduced NK cytotoxic activity, and higher viral loads, particularly in early time points; however, at later time points, pDCs seem to limit secretion of IFN-γ by NK cells (Krug *et al.* 2004, Swiecki *et al.* 2010). Further, although following pDC depletion expression of MHC-II by cDCs is considerably confined, the ability of cDCs to prime CD8$^+$ T cells is not affected (Swiecki *et al.* 2010). Similarly, in vesicular stomatitis virus (VSV), pDC depletion abolishes early IFN-α increase. However, dissimilar to CMV, pDCs promote accumulation and survival of antigen-specific T cells in VSV (Swiecki *et al.* 2010). In line with these findings, it has been shown that pDCs can induce cDC maturation and promote CD8$^+$ T cell clonal expansion by cDCs (Yoneyama *et al.* 2005).

In summary, in viral infections in various non-ocular tissues, pDCs play a vital role in viral encounters through production of type I IFNs and through modulating innate and adaptive immune responses, based on the offending agent and its route of entry. Considering the key role of pDCs in viral infections, below we review our understanding on their contribution to HSV-1 keratitis.

4.2.1.1. Ocular Viral Infections: As described above, classically, pDCs were acknowledged for their pivotal role in viral challenges (Cella *et al.* 1999, Siegal *et al.* 1999, Coccia *et al.* 2004, Krug *et al.* 2004, Lund *et al.* 2006, Smit *et al.* 2006, Swiecki *et al.* 2010). Thus, viral infections were among the first conditions in which the role of pDCs were investigated in ocular diseases. In this regard, in an early report, Kittan *et al.* assessed the distribution and function of pDCs in acute retinal necrosis caused by HSV or VZV in humans. They observed that in individuals with acute retinal necrosis, pDCs seem to display a lower frequency in the blood stream compared to controls. However, they observed low numbers of pDCs in the vitreous of one (out of two) of the examined patients, suggesting potential redistribution of pDCs to the site of inflammation. Further, although pDCs isolated from the peripheral blood of the individuals with acute retinal necrosis expressed higher levels of co-stimulatory molecules, their capacity to produce IFN-α was limited compared to controls (Kittan *et al.* 2007).

Considering constant exposure of the ocular surface to the environment and thus pathogens, shortly following the identification of resident corneal pDCs, their significance was investigated in viral infections of the cornea. In this regard, due to the importance of HSV-1 keratitis as the leading cause of infectious blindness in developed countries (Liesegang 2001), studies on the role of pDCs in corneal infections mostly focused on HSV-1 keratitis (Hu *et al.* 2013, Sendra *et al.* 2015, Sendra *et al.* 2016).

Dissimilar to other tissues, primary infection of HSV-1 in humans rarely accompanies clinical symptoms and signs in the cornea (Darougar *et al.* 1985, Liesegang *et al.* 1989);

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Typically, during primary ocular or mucosal infection, HSV-1 invades the sensory dendrites and is transferred to neuronal cell bodies in trigeminal ganglion (TG), where it remains in a dormant state. However, following the resolution of the primary infection, the dormant virus in TG can be re-activated by various stressors, traveling back to the cornea via sensory dendrites in a retrograde fashion, leading to recurrent epithelial keratitis manifested as corneal inflammation, neovascularization, scarring, perforation and in severe cases blindness (Liesegang 1999, Giménez *et al.* 2013, Rowe *et al.* 2013). Although the HSV-1 virus entry to TG is thought to follow a non-corneal HSV-1 infection, it may also arise from "front door" transmission through the cornea (Kaye *et al.* 1992, Kovacs *et al.* 2009, Shah *et al.* 2010).

To directly assess the contribution of pDCs in immune responses in HSV-1 keratitis, our group has examined how density of pDCs is altered during the course of acute HSV-1 keratitis. We observed that as early as 1 day following HSV-1 inoculation, pDCs are increased in both peripheral and central corneas compared with sham-inoculated controls and their increase tends to progress till day 6 post inoculation (Hu *et al.* 2013) (manuscript under review). This observation was further validated by flow cytometry, showing considerable increase in the density of $CD45^+$ $CD45R/B220^+$ $PDCA\text{-}1^+$ pDCs, which co-expressed CD11c, Ly49Q, Ly6C, and Gr-1, but are negative for CD11b, F4/80, Ly6G, CD3, and CD19 on day 3 following HSV-1 inoculation (manuscript under review). To evaluate how depletion of pDCs alters immune response to HSV-1 keratitis, transgenic BDCA-2-DTR mice which express simian DTR under the control of pDC specific BDCA-2 promoter (Swiecki *et al.* 2010) have been used. It has been shown that pDC depletion prior to HSV-1 inoculation is accompanied by deterioration of clinical severity of HSV-1 keratitis, enhanced infiltration of immune cells to the cornea, increased viral load in the cornea, and viral transmission to dLNs and TG (Hu *et al.* 2013, Sendra *et al.* 2017) (manuscript under review; Fig. 5). Further, it has been shown that pDC depletion is accompanied by reduced IFN-α levels in the cornea, and blocking TLR-9 in pDC-sufficient corneas prevents HSV-1 induced IFN-α response, suggesting an important role of pDCs signaling through TLR-9 in IFN-α responses in acute HSV-1 keratitis (manuscript under review). Nevertheless, the study does not clarify whether the observed deterioration of clinical severity of HSV-1 keratitis or enhanced tissue damage in pDC-depleted corneas is linked to reduced IFN-α secretion or potentially other properties of pDCs.

In contrast to the cornea, the periorbital skin, including eye lids, similar to skin covering other body sites, clinically manifests signs of primary HSV-1 infection (Darougar *et al.* 1985). Noteworthy, the skin is known to be devoid of resident pDCs during steady state, and thus, it might be postulated that the presence of tissue-resident pDCs in the cornea during steady state may, at least in part, explain how the cornea is preserved from signs of primary HSV-1 infection in humans. Nevertheless, this hypothesis needs to be further tested by evaluating immune response to skin tissue, with prior adoptive transfer of pDCs or alternatively, in murine corneas depleted from pDCs and inoculated with low dose of HSV-1, which is usually infective for skin.

The role of pDCs in mediating adaptive immune responses in HSV-1 keratitis has also been studied. It has been shown that following corneal HSV-1 inoculation, the density of pDCs increases remarkably in the dLNs, with a major shift towards mature ($MHC\text{-}II^+$) pDCs.

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Further, although the distribution of pDCs in subcapsular, paracortical, and cortical areas of the dLN is remained unchanged following corneal HSV-1 inoculation, their motility and displacement is enhanced in the dLNs (Sendra *et al.* 2016). Further, it has been reported that depletion of corneal pDCs in BDCA-2-DTR mice prior to HSV-1 inoculation is accompanied by alterations in the dLN cytokine milieu, leading to decreased density of Tregs (Sendra *et al.* 2017) (manuscript under preparation). Recently, it has been shown that during HSV-1 keratitis, Tregs may become unstable and can be reprogrammed to effector T cells. Such ex-Tregs harbor pathogenic properties and can propagate the severity of keratitis (Bhela *et al.* 2017). Recent studies highlight that pDCs not only favor generation of Tregs during HSV-1 keratitis, but they also prevent reprogramming of Tregs to pathogenic effector ex-Tregs. In this regard, it is shown that, local corneal depletion of pDCs is accompanied by enhanced density of ex-Tregs in the dLN as well as increased recruitment of ex-Tregs to the cornea *in vivo*. Further, *in vitro* experiments indicated that co-culture of pDCs with Tregs prolongs expression of Foxp3 in Tregs and diminishes their reprogramming to effector T cells (manuscript under review).

Our current knowledge on the role of pDCs in mediating innate and adaptive immune responses in HSV-1 keratitis is depicted in Fig. 5. Despite these findings, our knowledge is limited on how pDCs mediate several aspects of immune responses in HSV-1 keratitis. For instance, although it has been reported that following inflammation, a higher frequency of corneal pDCs express the proliferation marker Ki-67 (Schwarzenbacher *et al.* 2017), currently the contribution of extravasating pDCs from the blood versus the potential *in situ* proliferating resident pDCs and their respective functions following HSV-1 keratitis remains to be elucidated. Further, molecular mechanisms through which pDCs may prevent viral entry and transmission to TG are not studied. In addition, future experiments may reveal if pDCs may interact with other cells/structures in the cornea, such as epithelial cells, stromal cells, and corneal nerves to mediate the immune responses following exposure to HSV-1 and to re-establish homeostasis following resolution of the keratitis. Thus, further studies are needed to assess if the findings on the important role of pDCs in controlling HSV-1 keratitis can be generalized to other viral causes of keratitis. In this regard, studying the significance of pDCs in mediating immune response and clearing the virus in viral conjunctivitis and more importantly sight-threatening viral infections such as varicella zoster virus (VZV) keratitis or CMV retinitis, warrants further investigation.

4.2.2. Bacterial Infections—In addition to their pivotal role in viral infections, pDCs contribute to immune responses during bacterial infections. Early evidence on their potential role in bacterial infections was provided by Svensson *et al.*, who showed that upon stimulation with *Staphylococcus (S.) aureus*, pDCs increase and produce type I IFNs (Svensson *et al.* 1996). Later, it was shown that exposure to *S. aureus* and other bacteria, such as *Neisseria meningitides* and *Haemophilus influenza* may trigger secretion of cytokines, such as IL-6 and TNF-α by pDCs (Michea *et al.* 2013). Similarly, exposure to gram positive bacteria can enhance cytokine production by pDC and their capability to promote $CD4^+$ T cell expansion and proliferation (Raieli *et al.* 2019).

During *Listeria (L.) monocytogenes infection*, pDCs tend to accumulate in the lymph nodes and spleen and up-regulate expression of the co-stimulatory molecule CD86 and maturation

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marker MHC-II (Tam *et al.* 2006). pDC depletion leads to reduced levels of pro-inflammatory serum cytokines, including IFN-γ, IL-6, and IL-12p40, reduced pathogen load, and improved survival (Takagi *et al.* 2011). Further, it has been shown that although bone marrow pDCs encountered with *L. monocytogenes* produce IFN-α and IFN-β in a MyD88-dependent fashion *in vitro*, their contribution to the production of type I IFNs in *L. monocytogenes in vivo* is minimal (Stockinger *et al.* 2009). In line with these findings, depleting pDCs with anti-PDCA-1 antibody during *L. monocytogenes* infection does not affect type I IFNs levels (Solodova *et al.* 2011). Thus, in bacterial infections, pDCs may not serve as the main source of type I IFNs, but may play a pivotal role in mediating both innate and adaptive immune responses through other yet unknown mechanisms.

Protective effects of pDCs are also evident in *Citrobacter (C.) rodentium* bacterial colitis, where pDCs are increased in the spleen and infiltrate the colon. Systemic pDC depletion in *C. rodentium* results in overall poor health, necessitating euthanasia (Rahman *et al.* 2019). Although pDC depletion does not affect the density of infiltrating immune cells, $CD3^+$ $CD4^+$ Th cells, and Tregs, it is accompanied by increased pro-inflammatory serum cytokines, increased vascular permeability and higher bacterial burden, suggesting the importance of pDCs in conserving the architecture of mucosal barrier (Rahman *et al.* 2019).

The molecular mechanisms, through which pDCs sense and respond to bacteria, are still controversial. While a study suggested that production of IFN-α following exposure to *S. aureus* is not mediated through TLR-2 and may require TLR-7/TLR-9 activation (Parcina *et al.* 2008), other evidence suggests that secretion of IFN-α can be abolished via blocking TLR-2 (Raieli *et al.* 2019). Additionally, the secretion of other pro-inflammatory cytokines and up-regulation of co-stimulatory molecules by pDCs may be mediated through TLR-1 (Raieli *et al.* 2019).

In summary, bacterial infection or exposure to bacteria *in vitro* alters pDCs properties and enables them to alter the inflammatory milieu and to prime naïve T cells to differentiate into other T cell populations. However, unique features of the immune responses mediated by pDCs to various bacteria and modulating different T cell and B cell responses by pDCs in both mice and human remains to be elucidated. In ocular tissues, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus* species, and *Pseudomonas aeruginosa* are among the main causes of serious bacterial infections of the conjunctiva and cornea. Considering the role of pDCs in mediating immune response in various bacterial infections, it is worthwhile to study if and how pDCs may control potentially blinding bacterial infections.

4.2.3. Parasitic Infections—To date, few studies have studied the role of pDCs in parasitic infections, such as toxoplasmosis and malaria infections. Toxoplasmosis, caused by the obligate intracellular protozoan *Toxoplasma gondii*, is the most common cause of infective retinitis in immunocompetent patients. Following systemic *T. gondii* infection, through signaling of TLR-11, pDCs in the lymph nodes proliferate and acquire a mature state by up-regulating MHC-II and co-stimulatory molecules, suggesting an early role for pDCs in T cell activation (Pepper *et al.* 2008). Further, Koblansky *et al.* showed that while pDCs express both TLR-11 and TLR-12, the role of TLR-12 is more prominent in the induction of immune responses during toxoplasmosis, as exposure to profilin-like protein

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from *T. gondii* enhances production of IL-12p40 in pDCs. However, this effect is abrogated in pDCs isolated from TLR-12$^{-/-}$, but not TLR-11$^{-/-}$ mice (Koblansky *et al.* 2013). Further, in TLR-11$^{-/-}$ mice, depletion of pDCs succumb the relative resistance to toxoplasmosis, enhancing the mortality of mice upon *T. gondii* challenge (Koblansky *et al.* 2013). The effect of *T. gondii* on pDCs resembles exposure of these cells to IL-10, since both result in the abolishment of cytokine production, by regulating downstream effects of TLR-9 signaling, such as IRF7 and STAT3, thereby blocking IFN-α production (Pierog *et al.* 2018).

In summary, pDCs mediate various innate and adaptive immune responses to offending pathogens ranging from viruses to parasites. While in viral exposures pDCs mainly employ TLR-7 and TLR-9 for responding to the pathogen, their machinery for sensing other microorganisms is less known and may include other TLRs.

4.3. Role of Plasmacytoid Dendritic Cells in Autoimmunity and Sterile Inflammation

4.3.1. Sjögren's Syndrome—Sjögren's syndrome (SS) is a systemic autoimmune disease, which although primarily affects salivary and lacrimal glands, can also affect other organs (Zoukhri 2006). Initial evidence on the potential implication of pDCs in the pathogenesis of SS originated from genome-wide gene expression profiling of minor salivary gland in individuals with SS. The study demonstrated that in individuals with SS, IFN-inducible genes are up-regulated, suggesting that pDCs, as the main producers of type I IFNs, may participate in pathogenesis of SS (Gottenberg *et al.* 2006). Similarly, in monocytes of individuals with SS, a cluster of IFN-inducible genes are up-regulated, which together with higher expression of CD40 on pDCs in these individuals, suggests that pDCs may play a role in the pathogenesis of SS (Wildenberg *et al.* 2008). Further, the density of circulating pDCs is decreased in individuals with SS (Vogelsang *et al.* 2010) and while pDCs were not found in salivary glands of healthy individuals, they accumulated in the main or minor salivary glands in individuals with SS, signifying they are redistributed from circulation to affected tissues in SS (Gottenberg *et al.* 2006, Vogelsang *et al.* 2010).

Behavior of pDCs is altered in the course of SS. In fact, miRNome analysis of circulating pDCs has shown that pDCs isolated from individuals with SS exhibit distinctive expression of miRNAs involved in regulation of apoptosis, autophagy, and survival, compared with pDCs isolated from healthy controls (Hillen *et al.* 2019). Additionally, transcriptome of peripheral pDCs from individuals with SS patients are similar to pDCs stimulated by TLR-7, as both exhibit low ribosomal proteins expression (RPL11, RPL27 and RPS11) compared with pDCs isolated from healthy volunteers. Further, it has been shown that TLR-stimulated pDCs from individuals with SS produce remarkably higher levels of IFN-α and IFN-b compared with pDCs from healthy individuals, altogether, indicating that pDCs are activated during the course of the SS (Hillen *et al.* 2019). Further, pDCs are suggested to have an indirect role in B cell recruitment to minor salivary glands, and thus to contribute to the development of SS, as IFN-α secretion by pDCs promotes release of CXCL13 by macrophages, which in turn leads to the recruitment of B cells (Zhao *et al.* 2016). Studies also assessed alterations in the functions of pDCs during SS. *In vitro* studies found that pDCs can phagocytose autoantigens, which exist in apoptotic bodies of epithelial cells of human submandibular gland in individuals with SS. Exposure to these apoptotic particles led

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to the production of inflammatory cytokines such as IFN-α, IL-6, IL-8 and TNF-α by pDCs through TLR-7 and TLR-9. Additionally, it is known that sex hormones are altered in SS, with decreased estrogen and dihydrotestosterone levels. These sex hormones had no effect on TLR-7 and TLR-9 expression by pDCs, and did not alter their pro-inflammatory cytokine production *in vitro*; however, they were protective of the epithelial cells, reducing their apoptosis, and thus, limiting exposure of pDCs to autoantigens (Ainola *et al.* 2018)

In summary, pDCs accumulate in the salivary glands in the course of SS and exhibit characteristics of activation. However, it is not clear if they are in fact the initiators of the disease or if they contribute to the progress of the disease through secretion of type I IFNs and cytokines. Of note, lacrimal gland dysfunction is one of the hallmarks of SS, and it remains unknown how pDCs are altered in the lacrimal gland in the course of the disease and how they may contribute to the disease pathophysiology. Thus, further studies are necessary to evaluate the role of pDCs in the lacrimal gland in SS.

4.3.2. Systemic Lupus Erythematosus—Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by loss of tolerance to self-antigens, with a wide range of clinical manifestations (Dorner *et al.* 2019). Ocular involvement is common in individuals with SLE, affecting about one third of patients. In addition to its most common associate, Sjögren's syndrome (please see relevant section above), ocular manifestation of SLE range from involvement of ocular adnexa to vision-threatening retinal vasculitis to optic neuropathy (Palejwala *et al.* 2012, Shoughy *et al.* 2016, Silpa-archa *et al.* 2016). In SLE, several autoantibodies against self-molecules in the nucleus, cytoplasm, and cell surface, in addition to soluble molecules, such as IgG and coagulation factors, participate in the pathogenesis of the disease (Dorner *et al.* 2019). Due to the presence of a variety of autoantibodies, SLE can virtually manifest in any tissue. Neutrophil extracellular traps containing nucleic acid antigens, as well as apoptotic cells, may expose DNA and nuclear proteins that interact with autoantibodies to form immune complexes. pDCs harbor Fc receptors on their surface and are thus capable of engulfing immune complexes. Following internalization of immune complexes, fusion with endosomes allows the engagement of TLR-7 and TLR-9, mediating pathogenic production IFN-α, along with other cytokines such as TNF-α (Tian *et al.* 2007, Sakata *et al.* 2018, Smith *et al.* 2019). Secreted type I IFNs subsequently activate and sustain autoantibody generating B cells (Jego *et al.* 2005, Banchereau *et al.* 2006).

Early depletion of pDCs in SLE ameliorates the disease progression in mice, by limiting aberrant B cells and subsequent autoantibody generation (Rowland *et al.* 2014). Conversely, pDC repopulation results in reduced splenic weight, decreased autoantibodies, B cells and $CD4^+$ T cells. Thus, temporary pDC depletion results in favorable outcomes, suggesting a pathogenic role of pDCs, at least at the onset of SLE. In $Tcf4^{+/-}$Tlr7.tg mice, which develop a SLE-like disease, absence of pDCs leads to reduced splenomegaly, serum anti-RNA IgG levels, normalization of the density of peripheral $CD11c^+$ $MHC\text{-}II^{neg}$ cells, and increased survival. Therefore, lack of pDCs ameliorates the immune activation observed in the Tlr7.Tg SLE-like mice (Sisirak *et al.* 2014). Moreover, pDCs from mice with late SLE are unable to produce IFN-α upon TLR stimulation (Liao *et al.* 2015). Together, these results indicate that

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pDCs lose the ability to produce IFN-α in the course of SLE, and therefore, may not be necessary for disease progression.

In humans, circulating pDCs are increased in individuals with SLE as compared to healthy individuals (Jin *et al.* 2008), and have an increased ability to stimulate and expand T cells. Conversely, pDCs from healthy individuals are not capable of stimulating T cells, but are instead capable of inducing Tregs (Jin *et al.* 2010). Furthermore, pDCs during steady state can induce both Bregs ($CD24^{+}$ $CD38^{hi}$) that co-express IL-10 and plasmablasts through secretion of IFN-α and CD40 signaling. In healthy conditions, Bregs provide a negative feedback and reduce IFN-α production by pDCs via secretion of IL-10. However, during SLE, pDCs fail to induce the differentiation of Bregs and instead solely promote antibody-producing plasmablasts(Menon *et al.* 2016). Targeting the pDC specific receptor BDCA-2 with a humanized monoclonal antibody (24F4A) results in blocking pDC-mediated IFN-α expression in the serum of SLE patients (Gardet *et al.* 2019). Furthermore, injections of 24F4A in monkeys inhibit pDC activation by SLE-associated immune complexes (Pellerin *et al.* 2015). Application of BIIB059, another humanized monoclonal antibody against BDCA-2, which is currently under investigation in a phase II clinical study (NCT02847598), reduces skin damage and increases internalization of BDCA-2 in pDCs, which correlates with reduced levels of circulating IFN-α (Furie *et al.* 2019). Since IFN-α has been shown to be one of the pathogenic mediators during SLE, current clinical trials with anti-IFN-α antibodies are ongoing and are in phase III clinical trials (NCT01438489) (Furie *et al.* 2017).

In summary, although secretion of type I IFNs is necessary for immune response to pathogens, secretion of high levels of IFN-α by murine pDCs at the disease onset activates and sustains B cells to produce autoantibodies; however, the role of pDCs in disease progression is to be elucidated.

4.3.3. Rheumatoid Arthritis—Although pDCs seem to serve as culprit in SLE, this may not be the case in the majority of other autoimmune disease in which the role of pDCs has been investigated. Therefore, in order to provide a more balanced view of potential protective and destructive roles of pDCs in autoimmune conditions, we briefly review our current understanding of the role of pDCs in rheumatoid arthritis, a condition in which pDCs may play a protective role. Rheumatoid arthritis is a chronic disease that primarily affects joints, but can include vasculitis or other systemic comorbidities. It has been established that between 50% and 70% of individuals with rheumatoid arthritis share autoantibodies against citrullinated peptide and against IgG (rheumatoid factor) (Nell-Duxneuner *et al.* 2010, Barra *et al.* 2011). In the synovial milieu, a Th1/Th2 imbalance towards Th1 cells has been described in RA patients, and this imbalance is driven by pDCs, cDCs and B cells (McInnes *et al.* 2007). Despite the fact that pDCs can exert a Th1 response by producing IFN-α, the involvement of IFN-α production by pDCs during rheumatoid arthritis remains controversial (Nehmar *et al.* 2017). A recent study employed different strategies to deplete pDCs to explore the contribution of type I IFNs and pDCs in a mouse model of rheumatoid arthritis. To induce rheumatoid arthritis in this study, sera of K/BxN mice, which contain pathogenic antibodies against the ubiquitous protein glucose-6-phosphate isomerase, necessary to develop the majority of rheumatoid arthritis features (Monach *et al.* 2007), were injected into mice. Following induction of rheumatoid arthritis, $Ikaros^{-/-}$ mice, which lack peripheral

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pDCs (Allman *et al.* 2006), displayed lasting bone erosion and paw swelling, accompanied by an increased influx of immune cells, mainly neutrophils in the peri-articular tissues (Nehmar *et al.* 2017). Similarly, depletion of pDCs via different methods leads to increased paw swelling and serum levels of IL-6, but does not significantly alter histological findings (Nehmar *et al.* 2017). In another model of rheumatoid arthritis, depletion of pDCs exacerbates collagen-specific proliferation of T cells, autoreactive B cells, and disease pathology, evident by extensive synovial hyperplasia, cartilage degradation, and pannus invasion (Jongbloed *et al.* 2009).

In individuals with rheumatoid arthritis, the density of blood pDCs is decreased and pDCs possess an immature phenotype, defined by decreased expression of CD40L, CD80, CD83, CD86, and adhesion molecule, L-selectin (CD62L) (Jongbloed *et al.* 2006, Cooles *et al.* 2018, Nehmar *et al.* 2018). Further, in individuals with rheumatoid arthritis, pDCs are able to favor generation of IL-10-secreting Treg cells from allogeneic naïve $CD4^{+}$ $CD25^{neg}$ T cells cell (Kavousanaki *et al.* 2010). Peripheral pDCs show increased CCR7 (a key chemokine receptor for migration of DCs to lymph nodes), which is inversely correlated with pDC frequency (Cravens *et al.* 2007, Seth *et al.* 2011). In addition, the CCR7 ligand, CCL19/CCL21, is increased in synovial joints in rheumatoid arthritis, supporting migration of pDCs to the joints in the early course of the disease (Pickens *et al.* 2011). Taken together, the presence of pDCs seems to limit the immune response and reduce inflammation-induced tissue damage in rheumatoid arthritis, suggesting a beneficial role for pDCs in this condition (Jongbloed *et al.* 2009, Kavousanaki *et al.* 2010, Nehmar *et al.* 2017).

In summary, the role of pDCs in pathogenesis of autoimmune diseases depends on the nature of the disease. During early SLE, when IFN-α signature is prominent, pDCs may participate in the pathogenesis and progression of the disease. However, in rheumatoid arthritis, pDCs tend to promote tolerance, re-establish homeostasis, and thus, favor clinical outcome of the disease. In ocular diseases, it would be interesting to assess if pDCs contribute to pathogenesis of autoimmune conditions, such as uveitis. Of note, considering the important role of IL-23/IL-17 signaling pathway in induction of autoimmune uveitis (Zhong *et al.* 2020) and counter-regulatory role of Tregs in this process (Grégoire *et al.* 2016, Zhuang *et al.* 2017), it may be postulated that pDCs may contribute to the pathogenesis of autoimmune uveitis by mediating T cell priming and preferably inducing particular T cell responses. Findings of such studies may pave the way for novel therapies targeting immune responses and thus ameliorating clinical severity and sequela of the disease.

4.3.4 Sterile Inflammation—Homeostatic properties of pDCs are not confined to induction of tolerance to antigens as described in the following section. In fact, pDCs drive anti-inflammatory responses in various conditions. For example, in an acute immune-mediated liver injury model, pDC depletion is accompanied by severe liver injury, judged by increased serum aminotransferase levels, increased serum IFN-γ and IL-6 levels, as well as to decreased infiltration of Tregs to the liver (Koda *et al.* 2019). Along the same lines, adoptive transfer of pDCs results in decreased serum aminotransferase, IL-6, and MCP-1 levels, reduced generation of IFN-γ^{+} Th1 and Th17 effector T cells, enhanced generation of generation of Tregs through IL-35 and favors mice survival (Koda *et al.* 2019).

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In the ocular tissues, sterile inflammation occurs in response to contact lens wear, allergens, self-antigens, and mechanical, thermal and chemical traumas and burns. Among ocular tissues, the cornea is unique to study immune responses due to its accessibility and simplicity of clinical examination, as well as its immune and angiogenic privilege. To study immune responses in the cornea, sterile models of inflammation, including thermal cautery, alkali burn, and corneal suture placement have been widely used (Pfister *et al.* 1978, Williamson *et al.* 1987, Ormerod *et al.* 1989, Sano *et al.* 1995, Streilein *et al.* 1996, Hamrah *et al.* 2002, Cursiefen *et al.* 2004, Giacomini *et al.* 2014). Recently, it has been shown that depletion of corneal pDCs in BDCA-2-DTR mice prior to suture placement is accompanied by enhanced clinical opacity of the cornea as compared to controls, as well as augmented influx of inflammatory immune ($CD45^+$) cells in general, including neutrophils and macrophages (Sendra *et al.* 2014, Sendra *et al.* 2017). pDCs may also regulate adaptive immune response in the dLNs, as their depletion prior to corneal suture placement results in increased the density of $CD8^+$ T cells and B cells (Sendra *et al.* 2014). This model also demonstrates that the role of pDCs in mediating innate and adaptive immune response is not dependent on IFN-α, but rather might be mediated through other pathways, as neutralization of IFN-α using subconjunctival administration of anti-IFN-α antibody does not alter clinical severity of inflammation, density of recruited immune cells, or density of subpopulations of $CD4^+$ T cells in the dLN (Sendra *et al.* 2017) (manuscript under preparation).

Experimental DED is another instance in which the role of pDCs in sterile ocular surface inflammation is studied. In this regard, Stern *et al.* evaluated IFN-α production in the tears of feeble mice, which carry a mutation in Slc15a4, abrogating IFN-α and cytokine production relatively specially in pDCs (Blasius *et al.* 2010, Stern *et al.* 2013). They demonstrated that compared with wild-type mice, feeble mice show significantly lower amount of IFN-α in their tears, suggesting that pDCs are the major source of IFN-α during desiccating stress-induced experimental DED (Stern *et al.* 2013). Fig. 6 summarizes our current understating on significance of pDCs in sterile corneal inflammation.

In summary, pDCs may contribute to amelioration of inflammation in sterile inflammations. Thus, it is worthwhile to assess the role of pDCs in other sterile inflammatory conditions such as chemical and mechanical traumas and evaluate if they may be involved in homeostasis and supporting stems cells and regeneration of corneal epithelium following traumas. Further, although capacity of pDCs in secreting type I IFNs is well known, it is interesting to study if contribution of pDCs to inflammatory processes such as DED goes beyond secretion of type I IFNs and if so, how such functions of pDCs are regulated.

4.4. Role of Plasmacytoid Dendritic Cells in Tolerance

In addition to directing immune responses to pathogens and their involvement in pathogenesis of autoimmune diseases, pDCs are pivotal in inducing tolerance and suppressing inflammatory responses. In fact, pDCs provide a tolerogenic microenvironment, maintaining homeostasis, through various mechanisms, ranging from secretion of tolerogenic cytokines and growth factor, the development of Tregs in the thymus and peripheral tissues, to mediating T cell activities (de Heer *et al.* 2004, Martin-Gayo *et al.* 2010).

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In general, freshly isolated pDCs from human blood, unlike cDCs, express ICOS-L upon *in vitro* stimulation, through which they may prevent expansion of naïve CD4$^+$ T cells and augment generation of IL-10 producing Tregs (Ito *et al.* 2007). Further, upon stimulation with TLR-9 agonists, human peripheral blood pDCs shift cytokine production pattern of naïve CD4$^+$ T cells towards enhanced production of TGF-β and IL-10, and decrease secretion of IL-2. They also favor generation of Tregs, which can suppress autologous and allogeneic T cell proliferation in an Ag-nonspecific manner *in vitro* (Moseman *et al.* 2004). Dissimilar to cDCs, when stimulated pDCs are cultured with naïve CD8$^+$ T cells, they prime CD8$^+$ Tregs with poor secondary proliferative capacity and cytotoxic activity against allogeneic cells. These Tregs prevent allospecific proliferation of naïve CD8$^+$ T cells through secretion of IL-10 (Gilliet *et al.* 2002). Compared with cDCs, even stimulated pDCs exhibit lower capacity in promoting proliferation of allogeneic T cell. Further, pDCs also induce remarkable apoptosis in allogeneic CD4$^+$ T cells via Tregs (Tokita *et al.* 2008). Notably, it is shown that pDCs isolated from different tissues, for instance liver or spleen may display different tolerogenic capacities (Tokita *et al.* 2008). In the sections below, we briefly review the roles of pDCs in inducing tolerance to organ transplants, tumors, and oral antigens, as three main areas in which immune responses are currently under comprehensive investigations and where they may apply to respective ocular diseases.

4.4.1. Transplantation

4.4.1.1. Transplantation in Non-ocular Tissues: Recent studies have unraveled tolerogenic effects of pDCs in multiple organ transplants, such as heart, kidney, and hematopoietic stem cells, by highlighting their role in the generation or promotion of the functions of Tregs, as well as mediating T cell anergy (Abe *et al.* 2005, Ochando *et al.* 2006, Li *et al.* 2010, Rajasekar *et al.* 2010, Oh *et al.* 2019). For instance, in a rat model of heart transplantation, it has been shown that accumulation of pDCs in the graft is necessary for induction of tolerance and graft survival (Li *et al.* 2010). Dissecting the cellular players in mediating immune responses to grafts, Ochando *et al.* demonstrated that, in a murine model of cardiac transplant, pDCs capture alloantigens in the graft and egress to the dLN via blood circulation, where they induce generation of CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs. Furthermore, depletion of pDCs accelerates graft rejection in tolerized mice, while intravenous adoptive transfer of pDCs isolated from tolerized mice enhances Treg generation and favors graft survival (Ochando *et al.* 2006). Similarly, it has been shown that pDCs preferentially promote tolerogenic a function of CD8$^+$ Tregs on suppressing CD4$^+$ T cell proliferation, via a contact-dependent effect on CD8$^+$ Tregs (Li *et al.* 2010). In addition to favoring the generation of Tregs, pDCs may promote T cell anergy. In this regard, it has been shown that *ex vivo* CpG-ODN stimulated pDCs isolated from the bone marrow, are less efficient in inducing allogeneic naïve T cell proliferation compared to cDCs, which can be in part attributed to their expression of the co-inhibitory molecule PD-L1 (B7-H1) (Abe *et al.* 2005). Furthermore, intravenous adoptive transfer of pDCs induces non-specific hypo-responsiveness to challenge with donor or third-party irradiated splenocytes *ex vivo*, including reduced T cell proliferative, as well as IL-2 and IFN-γ secretory capacities (Abe *et al.* 2005). Interestingly, the addition of IL-2 to T cells, isolated from mice receiving pDCs, could not retrieve T cell proliferative capacity, indicating that pDC-induced hypo-responsiveness is not reversible (Abe *et al.* 2005). Moreover, pre-operative adoptive transfer

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of *in vitro*-propagated donor or third party pDCs significantly improves survival of fully MHC-mismatched heart transplants (Abe *et al.* 2005). Confirming these findings, it has been shown that adoptive transfer of mobilized donor pDCs prior to surgery can substantially promote heart allograft survival compared with subtypes of cDCs (Bjorck *et al.* 2005).

In kidney transplants, naïve pDCs isolated form syngeneic or accepted allogeneic transplants can generate Tregs from $CD4^+$ $CD25^{neg}$ T cells *in vitro*. Further, adoptive transfer of Tregs, generated *ex vivo* by co-culturing pDCs and $CD4^+$ $CD25^{neg}$ T cells, enhances graft survival (Oh *et al.* 2019). In contrast, pDCs are also implicated in immune responses to viral pathogens occurring following kidney transplantation. It has been shown that pDCs treated with conditioned media isolated from CMV-infected human kidney proximal tubular epithelial cells exhibit phagocytic activity and can increase $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production and thus, may contribute to kidney transplant rejection (Ruben *et al.* 2018). Tolerogenic effects of pDCs in transplantation are not confined to solid tissues. In fact, it has been shown that in patients undergoing HLA-matched hematopoietic stem cell transplant due to hematologic malignancies, higher graft pDC count is associated with increased risk of relapse and poor overall survival, persumably due to attenuation of graft-versus-leukemia effect (Rajasekar *et al.* 2010).

In summary, pDCs mediate the induction of tolerance to grafts and promote reestablishment of homesotasis in the transplanted tissue via generation of Tregs, induction of anergy, and production of anti-inflammatory cytokines, as well as by controling opportunistic infections. Consideirng that corneal trasnplntation is the most common solid tissue transplant with a high rate of rejection in high-risk individuals, it is thus necessesary to study the impact of pDCs in corneal transplntation and to assess if tolerogenic properties of pDCs can be utilized to improve transplantation outcomes. In the follwing section, we summarize our current knowledge on the significance of pDCs in corneal transplantation.

4.4.1.2. Corneal Transplantation: The role of resident professional APCs, such as cDCs in eliciting immune responses to corneal allografts is well documented (Hori *et al.* 2019). Immature APCs take up corneal alloantigens following transplantation and undergo maturation via upregulation of MHC-II and co-stimulatory molecules. They process the antigens and transfer them to the dLNs, where they prime naïve T cells to effector $CD4^+$ IFN-γ^+ Th1 cells. The effector Th1 cells then infiltrate the cornea, inducing immune rejection of the graft (Qazi *et al.* 2013, Amouzegar *et al.* 2016, Hori *et al.* 2019).

The role of pDCs in corneal transplantation is less studied compared to other solid organ transplants. In a recent study, Tahvildari *et al.* have shown that depletion of pDCs in recipient mice enhances graft opacity and infiltration of both innate and adaptive immune cells, in particular, $CD68^+$ macrophages and $CD3^+$ $CD4^+$ T cells (Tahvildari *et al.* 2017) (manuscript under preparation). Moreover, using ELISPOT assay, it has been reported that in pDC-depleted recipients, IFN-γ^+ T cells are increased in both direct and indirect allosensitization compared to controls. They further showed that pDC depletion prior to corneal transplantation is accompanied by increased frequencies of $CD4^+$ IFN-γ^+ Th1 cells and $CD4^+$ IL-17^+ Th17 cells, as well as decreased expression of CD25 among Tregs in the dLNs, leading to acceleration of immune rejection in pDC-depleted mice (Tahvildari *et al.*

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2017) (manuscript under preparation). Fig. 7 illustrates the role of pDCs in corneal transplantation.

Although current evidence suggests a critical role for pDCs in induction of tolerance to allogeneic transplants via suppressing the generation of effector T cells in the dLNs, several questions remain unanswered. For instance, it is not clear if pDCs mediate Treg induction in corneal transplantation or if they may affect several aspects of Treg biology, such as their survival, T suppressor activity, or fate. Furthermore, the molecular mechanism and signaling pathways through which pDCs exert their allosuppressive effects on subpopulations of T cells is not yet clear. Further studies may also unravel if tolerogenic properties of pDCs can be employed to induce specific tolerance of anergy in high-risk corneal transplantations and thus promote graft survival.

4.4.2. Allergic Diseases

4.4.2.1. Non-ocular Allergic Diseases: In this section, we will review our current understanding on the role of pDCs in the induction of tolerance to allergens, mainly to oral and airway allergens, and subsequently discuss potential contribution of pDCs to ocular allergies. Oral tolerance is the phenomenon by which tolerance is induced through the oral administration of antigens. This phenomenon has important implications for conditions such as allergy and asthma, and may, perhaps, be utilized in the future to promote tolerance in the context of autoimmunity (Weiner *et al.* 2011). While the role of Tregs in this context has received much attention, there are several studies that underscore the importance of pDCs in oral tolerance. Studies on mucosal-associated DCs revealed that $CD8\alpha^+$ DCs are promoters of Treg suppressive abilities. Further phenotypic characterization of these $CD8\alpha^+$ DCs indicated that both pDCs and cDCs are present in this population (Bilsborough *et al.* 2003, Fleeton *et al.* 2004). A series of *in vitro* proliferation assays indicated that $CD8\alpha^+$ pDCs are less capable of supporting T cell proliferation, and in fact, favor T cell suppression, even after maturation by exposure to CpG oligonucleotides (Bilsborough *et al.* 2003).

Additional reports have demonstrated that pDCs have an indispensable role in the induction of oral tolerance, as depletion of pDCs prevented tolerance. These reports investigated the contribution of liver-derived DCs to oral tolerance, and noted that the majority (60–80%) of liver-derived $CD11c^+$ $CD11b^{neg}$ $NK1.1^{neg}$ cells are pDCs (Goubier *et al.* 2008). As indicated by adoptive transfer and pDC depletion experiments, pDCs are able to limit the response of $CD4^+$ and $CD8^+$ T cells in response to oral challenge with 2,4-dinitro-1-fluorobenzene or ovalbumin. This effect is mediated by the ability of pDCs to induce anergy or deletion of $CD4^+$ and $CD8^+$ T cells in an antigen-specific manner (Goubier *et al.* 2008). The role of pDCs in tolerance induction is further confirmed by investigating the contribution of oral mucosa associated DCs. It is found that oral DC subsets, both cDCs and pDCs, are capable of promoting tolerance and are prone to polarizing naïve T cells towards Th1 or Treg phenotypes, in contrast to their splenic counterparts (Mascarell *et al.* 2008). Additional studies have led to the proposal of a two-step model for the induction of oral tolerance. In this first step, antigen-specific T cells are deleted or rendered anergic by pDCs, while simultaneously, pDCs promote the suppressive functions of Tregs. In the second stage,

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residual antigen-specific T cells are suppressed by Tregs upon antigen re-exposure, resulting in tolerance (Dubois *et al.* 2009).

Oral administration of probiotic *Lactobacillus gasseri* OLL2809, which induces oral tolerance, is accompanied by enhancing the ratio of pDCs as compared with cDCs in the lamina propria in the small intestine (Aoki-Yoshida *et al.* 2016). Moreover, it has been demonstrated that mesenteric LN-derived pDCs are potent inducers of $CD4^+$ $Foxp3^+$ Tregs, and neither inhibition of indoleamine 2,3-dioxygenase (IDO) nor blockade of B7 family members of co-stimulatory molecules can prevent generation of antigen-specific Tregs by pDCs *in vitro* (Uto *et al.* 2018). Rather, autocrine secretion of TGF-β mediates generation of such antigen-specific Tregs (Uto *et al.* 2018). In line with these *in vitro* findings, pDC-depleted mice fail to generate sufficient Tregs in the mesenteric LNs and fail to demonstrate protective tolerance to the antigen following feeding with OVA (Uto *et al.* 2018).

Findings from pre-clinical models have begun to be confirmed in humans (Hoffmann *et al.* 2006, Palomares *et al.* 2012). One such study investigated a variety of clinical parameters and white blood cell counts in a group of patients allergic to wheat. After the initial challenge with flour, there was reduced clinical response to the allergen upon subsequent challenge. This was associated with reduced numbers of circulating pDCs and Tregs, as well as reduced expression of MHC on DCs. This suggests that pDCs and Tregs are recruited to the site of challenge and promote a tolerogenic response (Hoffmann *et al.* 2006). Another report utilized the tonsils as a source of pDCs and tonsillar Tregs and peripheral blood as a source of naïve T cells from atopic and non-atopic individuals. Co-culture experiments revealed that pDCs are capable of inducing $Foxp3^+$ Tregs. Additionally, it was found that pDCs are decreased in atopic individuals compared to non-atopic individuals (Palomares *et al.* 2012).

Thus, in the context of oral tolerance, pDCs clearly have a central role. The studies described above also highlight that in each of the studied areas, pDCs have similar roles for the elimination of antigen-specific T cells and promoting the suppressive abilities of Tregs. These effects have proven to be relevant in animal models of allergy and atopy, and there are correlations between pDCs and Tregs in non-atopic humans, which is lost in atopic individuals. Altogether these findings suggest that pDCs represent a promising therapeutic target or, perhaps, cell-based therapy for the treatment of allergy and atopy. There is additional potential for utilizing pDC-driven oral tolerance to treat autoimmune diseases, however, this area requires further investigation.

Similar to oral tolerance, it is shown that pDCs promote tolerance to common allergens. In this regard, it has been shown that compared with control mice subjected to repeated exposure to ovalbumin aerosols, initial intratracheal administration of ovalbumin suppresses the airway inflammation, suggesting induction classic immunologic tolerance. However, only in mice undergoing depletion of resident pDCs in the lung, challenging mice with ovalbumin aerosols following initial intratracheal immunization, leads to eosinophilic infiltration around vessels and bronchi, goblet cell hyperplasia, and detection of ovalbumin-specific IgE in the serum, characteristic of asthma (de Heer *et al.* 2004). pDCs capture intratracheally administrated ovalbumin and migrate to dLNs, where among $ovalbumin^+$

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cells, pDCs tend to be more frequently ovalbumin$^+$. In stark contrast with cDCs, pDCs are not capable to induce ovalbumin-specific T cell proliferation and fail to secret higher amounts of pro-inflammatory cytokines following co-culture with T cells. In fact, pDCs induce differentiation of Tregs with suppressing effects on antigen-specific T cell proliferation. Adoptive transfer of pDCs pulsed with ovalbumin prior to sensitizing mice with intraperitoneal injection of alum- ovalbumin and subsequent challenge with ovalbumin aerosols significantly inhibits inflammation in the airways and T cell cytokine production. Thus, pDCs are vital for inducing tolerance to allergens and preserving tolerance to inert antigens (de Heer *et al.* 2004).

4.4.2.2. Ocular Allergic Diseases: In ocular allergies, in an early study summarized above, it was shown that pDCs, which were rarely observed in the conjunctiva during steady state, tended to significantly increase in this tissue following allergen challenge, reaching higher numbers than cDCs (Ohbayashi *et al.* 2007). However, the significance of these cells in the pathogenesis of allergic conjunctivitis warrants further investigation. In light of the essential role of pDCs in maintaining and inducing tolerance to oral antigens, as well as the pivotal role of pDCs in preventing allergic reactions in the respiratory system, as reviewed above, it might be postulated that pDCs promote tolerance to allergens on the ocular surface and thus diminish disease severity in individuals with allergic conjunctivitis.

Further studies on humans and murine models of allergic conjunctivitis may evaluate if the phenotype of pDCs and their tolerogenic properties are altered in allergic conjunctivitis and if so how such alterations can be reverted to promote tolerance and decrease severity of the disease. Further, it is intriguing to study if pDCs can be employed to induce tolerance to allergens after induction of immune response due to prior exposure to allergens and thus can be used to desensitize individuals to specific allergens and thus treat allergic conjunctivitis.

4.4.3. Tumors—Within the tumor microenvironment, pDCs have been found to infiltrate primary and metastatic tumors, as well as peri-tumoral tissues in multiple malignancies, including breast, ovarian, head and neck, gastric, liver, lung cancers, malignant melanomas, and lymphomas (Facchetti *et al.* 1989, Hartmann *et al.* 2003, Vermi *et al.* 2003, Kutzner *et al.* 2009, Conrad *et al.* 2012, Faget *et al.* 2012, Sawant *et al.* 2012, Asprod *et al.* 2013, Huang *et al.* 2014, Pedroza-Gonzalez *et al.* 2015, Sorrentino *et al.* 2015). Considering the well-known anti-tumor effects of IFN-α, studies aimed to evaluate if pDCs promote anti-tumoral immune responses through their secretion of IFN-α. However, tumor-associated pDCs fail to effectively produce type I IFNs (Zou *et al.* 2001, Hartmann *et al.* 2003, Labidi-Galy *et al.* 2011, Sisirak *et al.* 2012, Le Mercier *et al.* 2013, Dey *et al.* 2015, Terra *et al.* 2018), at least in part, due to secretion of immunomodulatory molecules by tumor cells, such as IL-10 and TGF-β (Bekeredjian-Ding *et al.* 2009, Sisirak *et al.* 2013, Bruchhage *et al.* 2018).

Although tumor-associated pDCs have a limited IFN-a production capacity, they do promote tolerance by suppressing T cell proliferation, cytotoxic activity, and IFN-γ secretion *in vitro* (Wei *et al.* 2005). Further, based on depletion studies, pDCs promote IL-10 secretion by CD4$^+$ T cells (Dey *et al.* 2015) and favor the accumulation of myeloid-derived suppressor cells in tumors (Sawant *et al.* 2012). In addition, pDC depletion leads to decreased density of Tregs in the tumor and metastases, as well as attenuated suppressive capacity of existing

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Tregs, suggesting a central role of pDCs in promoting Tregs (Sawant *et al.* 2012, Dey *et al.* 2015). Furthermore, pDCs isolated from tumor-draining LNs are shown to express IDO, and can activate suppressor activity of resting Tregs on $CD4^+$ and $CD8^+$ T cells in an IDO-dependent manner (Sharma *et al.* 2007). In addition to promoting Tregs, pDCs express PD-L1, which via interacting with PD-1 on T cells, limits proliferation and cytotoxic activity of both $CD4^+$ and $CD8^+$ T cells, as well as the cytolytic activity of NK cells (Ray *et al.* 2015). Expression of Granzyme B by pDCs can also regulate T cells, as secreted Granzyme B can inhibit $CD4^+$ T cell expansion (Jahrsdorfer *et al.* 2010). Similarly, tumor-associated pDCs express ICOS ligand (ICOS-L), which is necessary for survival and proliferation of $ICOS^+$ Tregs (Conrad *et al.* 2012, Faget *et al.* 2012). Further, via ICOS/ICOS-L signaling, stimulation of pDCs with tumor lysate induces $CD4^+$ $Foxp3^{neg}$ IL-10^+ Tregs from naïve $CD4^+$ T cells, which exhibit potent suppressive effects on T cell expansion (Pedroza-Gonzalez *et al.* 2015).

Although pDCs generally provide a tolerogenic microenvironment for tumors, upon stimulation, they are able alter these tolerogenic properties. For instance, stimulation of naïve pDCs through TLR-7 or TLR-9 enhances immune responses to tumors by multiple mechanisms: (1) enhancing direct cytotoxic activity of pDCs via enhancing their expression of Granzyme B and TRAIL (Drobits *et al.* 2012, Kalb *et al.* 2012, Wu *et al.* 2017); (2) stimulating expression of TRAIL, CD69, and IFN-γ by NK cells (Lelaidier *et al.* 2015), (3) cross priming and activating $CD8^+$ T cells and Th17 T cells (Lou *et al.* 2007, Liu *et al.* 2008, Guery *et al.* 2014), and (4) augmenting infiltration of NK cells and $CD8^+$ T cells in tumors (Liu *et al.* 2008, Le Mercier *et al.* 2013, Guery *et al.* 2014, Wu *et al.* 2017). Thus, stimulation of pDCs may alter intrinsic pDC functions and deserve further studies as a therapeutic strategy.

In summary, pDCs generally favor immune irresponsiveness to tumors via directly inhibiting T cells and NK cells or through promotion of Tregs. Nevertheless, pDCs may also affect other aspects of tumor biology, including direct effects on tumor cell proliferation, secretary functions, migration, invasion, metastasis as well as metabolism and angiogenesis in tumors, all of which warrant further studies. In ocular tissues, it is important to assess if pDCs play a similar tolerogenic role through directing suppressive immune responses for ocular neoplasia, such as in choroidal or conjunctival melanomas.

4.5. Plasmacytoid Dendritic Cell Function in Graft-Versus-Host Disease

Whereas transplant rejection occurs due to a host-mediated immune response, graft-versus-host disease (GVHD) reflects the opposite scenario, in which donor T cells primed by either donor or host APCs induce an immune response against the host. GVHD affects many organs. In particular, it may involve ocular tissues. Among ocular manifestations, DED is the most common presentation of ocular GVHD following HSCT (Munir et al. 2017). Our knowledge on the significance of pDCs in pathogenesis of ocular involvement of GVHD is limited and therefore, warrants detailed studies. However, we herein discuss our understanding on the role of pDCs in this disease.

In one of the early studies addressing the role of pDCs in GVHD, peripheral blood samples from individuals that had undergone hematopoietic stem cell transplantation (HSCT) were

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acquired and their immune cell subsets were profiled (Clark *et al.* 2003). This study found that pDC density in the peripheral blood of individuals with chronic GVHD are higher compared to control individuals who had undergone HSCT but did not develop GVHD (Clark *et al.* 2003). However, this difference is not due to an increase in pDCs in the GVHD group, rather it is due to decreased pDCs in control HSCT group without GVHD, since in the GVHD group, density of pDC in peripheral blood were comparable to healthy volunteers (Clark *et al.* 2003). In a murine model of GVHD, investigators irradiated MHC-II-deficient mice and reconstituted the APC populations (either pDCs, cDCs, or B cells), followed by T cells, showing that pDCs are capable of priming alloreactive T cells to induce GVHD (Koyama *et al.* 2009). A caveat to this study however, is that this is an artificial model, and it is unclear if pDCs would have the same effect following reconstitution with all APC populations. An additional study revealed that depletion of host-derived cDCs, pDCs, or B cells was insufficient to prevent the onset of GVHD (Li *et al.* 2012).

While it may be tempting to speculate that pDCs may thus be pathogenic, the possibility that pDC may promote tolerance cannot be excluded. In fact, there is a growing body of direct evidence that pDCs are protective in GVHD. One such study utilized a murine model of GVHD, where irradiated mice were reconstituted with STAT1$^{-/-}$ bone marrow, resulting in expanded pDC and Treg populations and GVHD resistance. Additionally, depletion of pDCs after reconstitution with STAT1$^{-/-}$ bone marrow reversed this effect (Capitini *et al.* 2014). Further, CCR9^{+} pDCs, which constitute the majority of pDCs in the dLNs, have potent tolerogenic capabilities, as they can effectively induce Tregs, which in turn inhibit CD4^{+} T cell proliferation considerably (Hadeiba *et al.* 2008). In addition, adoptive transfer of allogeneic CCR9^{+} pDCs is protective in GVHD, as it leads to decreased priming of naïve donor T cells towards IFN-γ^{+} Th1 and Th17 effector T cells in the dLNs and spleen, as well as to an enhanced density of CD25^{+} Foxp3^{+} Tregs in the dLN, with beneficial effects on animal survival (Hadeiba *et al.* 2008). In a large clinical study, multivariable analysis indicated that after adjustment for several factors, recipients of higher number of bone marrow pDCs showed improved 3-year survival, fewer deaths due to GVHD as well as rejection (Waller *et al.* 2014). Interestingly, the study did not observe a similar protective role for higher number of pDCs in grafts in individuals receiving granulocyte colony stimulating factor-mobilized peripheral blood HSCT. In follow-up studies, in a murine model of lethal GVHD, it was then confirmed that transplantation of bone marrow pDCs considerably improved survival, potentially due to secretion of IL-12, a capacity which was limited in pDCs isolated from the spleen following treatment with granulocyte colony stimulating factor (Hassan *et al.* 2017). In addition to potential differences in the IL-12 secretion capacity, other differences between bone marrow and mobilized peripheral blood pDCs may underlie this observation. For instance, bone marrow pDCs may contain higher numbers of less mature precursor pDCs with distinct antigen presentation capacity or chemokine receptor repertoire. In this regard, it has been shown that mobilized peripheral blood pDCs expressed higher levels of CCR7, lymph node homing receptor for pDCs, but lower levels of L-selectin (CD62L), CXCR3, and CCR9, which may facilitate homing of pDCs to inflamed tissues (Hosoba *et al.* 2014). Further studies have shown that an increase in pDC density in donor bone marrow has a protective effect by limiting GVHD (Hassan *et al.* 2019). In this regard, it has been shown that expansion of the pDCs *in vivo* by treating

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donor mice with FLT3-L, prior to reconstitution of irradiated recipient mice, leads to improved engraftment and protection from GVHD (Hassan *et al.* 2019). As such, increasing pDC numbers prior to bone marrow reconstitution may improve patient outcomes through their tolerogenic nature and capacity to induce Tregs. As expansion of pDCs in the prior study was done in vivo, it is interesting to study if ex vivo treatment of donor bone marrow cells with FLT3-L may yield a similar effect, or alternatively, supplementing the treatment regimen in recipients of HSCT with FLT3-L may promote engraftment.

In summary, pDCs are considered multifaceted cells of the innate immune system with diverse immune functions (Swiecki *et al.* 2015). While they were originally appreciated for their potent capacity in producing type I IFNs, our current knowledge suggests they are crucial implementer of tolerance and ameliorate inflammation.

4.6. Role of Plasmacytoid Dendritic Cells in Neuroprotection

The cornea is the most densely innervated tissue in the body, with approximately 300–600 times higher nerve density compared to the skin (Rozsa *et al.* 1982). Corneal nerves are in majority sensory, and arise from the ophthalmic branch of trigeminal nerves. Anatomically, they can be can be observed as the subbasal nerve plexus, the most densely innervated region of the cornea, which runs parallel to the superficial corneal surface between the Bowman’s layer and the basal epithelium and the stromal plexus, which consists of thicker nerve fiber bundles in the corneal stroma (Millodot 1984, Marfurt *et al.* 1993, Muller *et al.* 1996, Muller *et al.* 1997, Al-Aqaba *et al.* 2010, Marfurt *et al.* 2010, Belmonte *et al.* 2017, Cruzat *et al.* 2017). In addition to their vital role in initialing the corneal blink reflex and stimulating tear production, recent studies suggest that they play a crucial role in the development of multiple ocular surface diseases, including DED and neurotrophic keratopathy (Bonini *et al.* 2003, Dastjerdi *et al.* 2009, Hamrah *et al.* 2010, Hamrah *et al.* 2013, Hamrah *et al.* 2016, Mo *et al.* 2017, Neelam *et al.* 2018, Al-Aqaba *et al.* 2019, McKay *et al.* 2019). Constant exposure of the cornea to the external environment in the form of chemical irritants and pathogens, poses hazards to this intricate innervation. Further, similar to other peripheral nerves, corneal nerves need constant trophic support, such as members of neurotrophins family including NGF, BDNF, NT-3, NT-4/5 as well non-neurotrophin growth factors such as GDNF, neurturin, artemin, persephin, PEDF, NPFF and neuropoetic cytokines for their maintenance, proper function, or regeneration following injury (Daniele *et al.* 1992, Lambiase *et al.* 1998, Bonini *et al.* 2000, Kerschensteiner *et al.* 2003, Reichard *et al.* 2014, Dai *et al.* 2015, He *et al.* 2015, Razavi *et al.* 2015, Zhou *et al.* 2015). However, it is known that inflammatory conditions, as well as common surgical interventions such as cataract surgery, keratorefractive surgeries or keratoplasties, can result in at least partial corneal denervation (Wilson *et al.* 2001, Savini *et al.* 2004, Hamrah *et al.* 2010, Cruzat *et al.* 2011, Kurbanyan *et al.* 2012, Hamrah *et al.* 2013). Despite these insults, corneal nerves have a marked capacity for regeneration (Muller *et al.* 2015). Peripheral nerves are dependent on cues and survival signals from the tissues, which they innervate. Within the cornea, it remains to be determined which cell type(s) are responsible for this signaling. However, considering the well-studied roles for immune cells in wound healing and tissue repair/remodeling, exploring a potential contribution of immune cells in providing trophic support for corneal nerve is warranted. Early evidence on the potential communication of corneal

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nerves and pDCs stems from confocal microscopy of whole-mounted cornea labeled for βIII-tubulin (pan-neuronal marker), CD45, and PDCA-1 for visualizing corneal nerves (βIII-tubulin^{+}) and pDCs (CD45^{+} PDCA-1^{+} cells). As demonstrated in Supplementary Video 2, confocal micrographs reveal that corneal pDCs are present within the anterior stroma, in close association with the corneal nerves, suggesting that pDCs may contribute to neuro-immune crosstalk (Zheng *et al.* 2010) (manuscript under review).

Recently, the significance of resident corneal pDCs in homeostasis of corneal nerves has been investigated. It has been reported that depletion of corneal pDCs in naïve BDCA-2-DTR mice leads to an abrupt and robust degeneration of the corneal nerves, evident as early as one day following pDC depletion, and continues to progress while pDCs remain depleted, as determined by confocal microscopy on corneal whole-mounts stained with βIII-tubulin. Further, depletion of corneal pDCs is accompanied by reduced corneal sensitivity. These observations suggest that pDCs are indeed crucial for the maintenance and health of corneal nerves *in vivo* (Jamali *et al.* 2015). Analysis of TG neurons following corneal pDC depletion, has indicated an upregulation of several neurodegenerative markers, including tau and calpain-1, suggesting that depletion of pDCs and the ensuing nerve loss is at least in part mediated by tau oligomer neurotoxicity and subsequent calpain activation (Kenyon *et al.* 2019). More interestingly, pDCs can induce nerve regeneration as their repopulation, following initial depletion, is accompanied by nerve regeneration (Jamali *et al.* 2015). Direct neurotrophic properties of pDCs are shown in co-culture of TG neurons with freshly isolated splenic pDCs, which exhibited longer neurite outgrowth and expression of higher levels of neuroregenerative markers compared with TG neuron monocultures (Jamali *et al.* 2015). Further, pDCs exert their *in vitro* neurotrophic molecules through secretion of nerve growth factor (NGF; manuscript under preparation). Fig. 8 demonstrates the neuroprotective role of corneal pDCs.

Interestingly, the role of corneal cDCs in diabetes, as a neurotrophic corneal condition, has recently been investigated. It is shown that in diabetic mice, cornea encompasses less nerves and also hosts fewer cDCs. Further, it was reported that depletion of cDCs in CD11c-DTR-GFP mice, delays corneal nerve regeneration following nerve injury, which can be rescued by exogenous administration of CNTF, suggesting that cDCs may facilitate corneal nerve regeneration after wounding through secretion of neurotrophic molecule CNTF (Gao *et al.* 2016). Nevertheless, in contrast to pDCs, depletion of cDCs has not been reported to lead to corneal nerve degeneration during steady state.

Despite the reported neurotrophic properties of corneal pDCs, as a novel function of pDCs, our understating is limited as to whether resident pDCs in other ocular tissues or other body sites may also display neurotrophic properties. Further, it would be important to assess how pDCs are attracted towards the subbasal nerves in the cornea and how production of NGF in pDCs is regulated in this tissue. Also, considering the widely acknowledged trophic effects of the corneal nerves for epithelium, studies on role of pDCs on epithelial integrity may be important, as persistent epithelial defects, which may in turn facilitate access of pathogens, predisposes the cornea to infectious keratitis.

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4.7. Role of Plasmacytoid Dendritic Cells in Vasculature Integrity and Angiogenic Privilege

Significant role of infiltrating innate immune cells, in particular macrophages and cDCs, in promoting neovascularization has been appreciated for decades (Ribatti *et al.* 2009, Bruno *et al.* 2014). As mentioned above and illustrated in Fig. 4B, in our initial observations on the presence of pDCs in the conjunctiva and limbus, we reported that pDCs in a transgenic DPE-GFP×RAG1$^{-/-}$ mice with GFP-tagged pDCs (Iparraguirre *et al.* 2008) accompany limbal blood vessels at a high density compared to their densities in the conjunctiva and cornea (Jamali *et al.* 2020). Intravital multiphoton microscopy of GFP-tagged pDCs in the limbal region of the cornea demonstrates that during steady state (Supplementary Video 1) (Jamali *et al.* 2020) and in suture-induced neovascularized corneas, pDCs engulf limbal vessels (Supplementary Video 3). More detailed assessment of the pDCs shows that these cells are not statically residing by limbal vessels, rather they actively interact with the vasculature, for instance by extending their stellates around the newly-formed vessels (Supplementary Video 3; magnified region of interest in Supplementary Video 4). In addition to pDCs accompanying vessels, a fraction of pDCs also dynamically patrol intravascular spaces (Supplementary Video 5). Thus, collectively, our observations further suggest that pDCs may play a role regulating vasculature, in particular during dynamic process of inflammatory neovascularization, leading us to explore the hypothesis that pDCs, as another innate immune cell population, may also play a role angiogenesis.

In order to explore the potential role of pDCs in regulating neovascularization, we depleted pDCs locally in the cornea in transgenic BDCA-2-DTR mice. We reported that depletion of pDCs during steady state is accompanied by breakdown of angiogenic privilege (Jamali *et al.* 2016). Similarly, local depletion of pDCs, enhanced corneal neovascularization following suture placement (Jamali *et al.* 2016). Upon dissecting the molecular mechanisms through which pDCs may contribute to corneal angiogenic privilege and prevent corneal neovascularization induced by suture placement, it has been reported that pDCs secret a wide range of anti-angiogenic (angiostatic) molecules, including endostatin, thrombospondin (TSP)-1, platelet factor (PF)-4/CXCL4, and tissue inhibitor of metalloproteinase (TIMP)-3, and can thus inhibit endothelial cell proliferation *in vitro* (Jamali *et al.* 2016, Harris *et al.* 2018, Harris *et al.* 2019). Fig. 9 illustrates the significant role of pDCs in corneal angiogenic privilege.

4.8. Therapeutic Potential of Plasmacytoid Dendritic Cells

Considering the tolerogenic, anti-inflammatory, anti-angiogenic, and neuroprotective properties of pDCs, it is worthwhile to assess their potential in treating various ocular conditions in which inflammation, neovascularization, or nerve degeneration play key roles in their pathogenesis. These conditions may range from neovascular ocular diseases, including age-related macular degeneration, retinopathy of prematurity, diabetic retinopathy, corneal traumas, to inflammatory diseases such as microbial keratitis, uveitis, and endophthalmitis, and neurodegenerative conditions such as neurotrophic keratopathy. Potential therapeutic use of pDCs can be examined via different approaches, including systemic or local adoptive transfer of syngeneic naïve or *ex vivo*-stimulated pDCs, or administration of their rich culture supernatant. In this regard, the potential efficacy of local

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adoptive transfer of naïve pDCs isolated from murine spleen in enhancing corneal nerve regeneration following nerve damage has been assessed, as has been their efficacy in preventing corneal neovascularization, and treating acute HSV-1 keratitis (Jamali *et al.* 2017, Sendra *et al.* 2017, Jamali *et al.* 2019).

In this regard, a technique for transferring small number of pDCs locally to the cornea has been recently proposed (manuscript under review). In this method, following isolating pDCs from spleen or bone marrow of mice, the central cornea epithelium is mechanically removed via an Algerbrush and pDCs are locally transferred to the cornea using a fibrin sealant. As depicted in Fig. 10, transferred pDCs can be detected in the cornea following the procedure, indicating feasibility of the procedure in transferring pDCs. To assess the significance of local adoptive transfer of pDCs in enhancing nerve regeneration, paracentral/central corneal nerves were severed by using a trephine and following debridement of central cornea, pDCs were adoptively transferred locally to the cornea using fibrin sealant. Local adoptive transfer of pDCs enhances NGF levels and corneal nerve regeneration compared with application of fibrin sealant without cells or adoptive transfer of $CD11b^+$ myeloid cells (mainly containing cDCs and macrophages) control groups (Jamali *et al.* 2017). Similarly, adoptive transfer of splenic pDCs diminishes corneal neovascularization induced by suture placement compared with application of fibrin sealant without cells or adoptive transfer of $CD11b^+$ myeloid cells (Jamali *et al.* 2019). Moreover, pre-loading the cornea with pDCs locally 24 h prior to inoculation of HSV-1, is accompanied by less corneal opacity and viral load by enhancing the levels of anti-viral IFN-α and anti-inflammatory TGF-β (Sendra *et al.* 2017). Collectively, these observations suggest that local adoptive transfer of pDCs can suppress sterile and infectious corneal diseases.

Although trials in therapeutic application of pDCs to prevent corneal neovascularization and enhance corneal nerve regeneration in mice are promising, several concerns need to be considered in future studies. First, in these studies, the corneal epithelium was mechanically removed to facilitate migration of pDCs into the corneal stroma. Considering that mice do not have a Bowman’s layer, feasibility of locally transferring pDCs to the cornea might be more challenging for humans with thicker corneal epithelium and the presence of a protective Bowman’s layer as barriers. Similarly, feasibility of systemic or local adoptive transfer of pDCs to choroid and retina needs to be elucidated. More importantly, potential long-term side effects of systemic and local adoptive transfer of pDCs need to be carefully examined. While pDCs may show initial protective effects, considering their marked plasticity, it is not yet clear if adoptively transferred pDCs may alter their properties in the inflammatory microenvironment they are introduced to. This concern warrants further attention in the light of studies which show bone marrow pDCs may give rise to cDCs with pro-inflammatory properties during inflammation (Zuniga *et al.* 2004, Liou *et al.* 2008). Nevertheless, using blood or splenic pDCs that are shown to have less plastic capacity (Zuniga *et al.* 2004) may dampen this shortcoming. On the flip side, considering the tolerogenic property of pDCs, adoptively transferred pDCs may interfere with immune surveillance against tumors, facilitating development of primary tumors in ocular tissues or elsewhere, progression of cancers in remission, or development of secondary tumors.

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5. Future Directions

5.1. Life Cycle of Plasmacytoid Dendritic Cells

Life cycle and longevity of pDCs is an aspect of their biology, which deserves detailed exploration, in particular in peripheral tissues during steady state. While our current knowledge suggests that pDCs leave the bone marrow following terminal development, they may alter their phenotype and may convert to cDCs under certain conditions *in vitro* and *in vivo* (Grouard *et al.* 1997, O'Keeffe *et al.* 2003, Zuniga *et al.* 2004, Liou *et al.* 2008). It is worthwhile to dissect the molecular signaling that derives such conversion and also it is interesting to evaluate if other immune cells, such as their closet counterparts, cDCs, may convert to pDCs during the steady state or following inflammatory stimuli such as viral challenges. Based on our current understandings, pDCs in secondary lymphoid tissues, such as in the spleen, have been shown to be long-lived, with low proliferative capacity judged by lower expression of proliferative marker, Ki-67 as well as BrdU incorporation, compared with cDCs (O'Keeffe *et al.* 2002, Liu *et al.* 2007). Further, it has been shown that upon irradiating one of the parabiont pairs, pDCs in the secondary lymphoid tissues are replenished from the other parabiont partner, suggesting contribution of blood-derived pDCs or pDC precursors in repopulating the pool of pDCs in the secondary lymphoid organs (Liu *et al.* 2007). Nevertheless, it needs to be elucidated if pDCs in peripheral tissues such as lung, kidney, and ocular tissues also follow a similar pattern. Further, our knowledge is limited about the longevity of the resident pDCs in the ocular tissues and if they repopulate these tissues from blood pool, *in situ* proliferation, or potential precursors residing in these tissues.

5.2. Molecular Regulation of Plasmacytoid Dendritic Cell Function

Considering that production of type I IFNs has been the major focus of the studies on pDCs for several decades, it is not surprising that our knowledge on regulation of pDC functions has been predisposed to unravel how pDCs receive danger signal to produce type I IFNs in various viral and bacterial infections, how their IFN production machinery is assembled, and how IFN secretion is mediated. Considering the clinical importance of various viral, bacterial, and parasitic infections of the conjunctiva, cornea, and retina and feasibility of clinical and pathological examinations on these tissues, future studies can use these ocular tissues to assess the molecular mechanisms through which pDCs are activated upon exposure to pathogens. Nevertheless, considering the recently explored versatile immune and non-immune functions of pDCs such as promoting Tregs, neuroprotection, and anti-angiogenic properties, it is of particular interest to evaluate how these functions are regulated and if such regulation is dependent or independent of pathways that mediate IFN production in pDCs.

One approach to explore potential molecules that may regulate pDCs relies on identifying cell surface and intracellular receptors that are expressed by pDCs. In this regard, one set of candidates are currently known pDC markers which are coupled with intracellular signaling molecules, such as Ly6C and Siglec-H in mice and BDCA-2 and ILT-7 in humans. Another set of candidates include receptors, which are expressed by other immune cells, in particular by innate immune cells more close to pDCs, such as their classical counterpart, cDCs and

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macrophages. In this regard, purinergic receptors might be of particular interest. To date, four receptors have been reported which bind to ATP and its derivatives with varying specificities and affinities and all have been widely studied on other immune cells. ATP is typically found at negligible concentrations within the extracellular space, however, upon cellular injury, ATP or its related catabolites leaks out into the extracellular milieu (Van Belle *et al.* 1987, Pedata *et al.* 2001). This serves as a potent danger signal that attracts and activates immune cells. Similar to immune cells, purinergic signaling within the central nervous system leads to activation and migration of microglia and promotes the release of neurotrophic factors from microglia and astrocytes. Interestingly, it is shown that freshly isolated peripheral blood pDCs express adenosine receptor A1 and upon *ex vivo* stimulation, these cells downregulate adenosine receptor A1 and instead express adenosine receptor A_{2a} (Schnurr *et al.* 2004). Further, it has been shown that adenosine receptor A1 and A_{2a} may differentially regulate pDCs, since stimulation of adenosine receptor A1 in freshly stimulated pDCs serves as a potent pDC chemoattractant, while signaling through adenosine receptor A_{2a} in stimulated pDCs is coupled with reduced production of pro-inflammatory molecules IFN-α, IL-6, and IL-12 (Schnurr *et al.* 2004). Additionally, it has recently been shown that pDCs express members of ATP-gated P2X receptor cation channel family, namely, P2rx4 and P2rx7 and extracellular ATP signaling through P2rx7 may induce apoptosis in pDCs, another instance which suggests purinergic reporters may regulate pDC behavior (Furuta *et al.* 2017).

Another interesting set of receptors that may play a role in regulating pDC functions are receptors for neurotrophic molecules. Currently, it has been shown that pDCs express p75NTR, which can regulate functions of pDCs in asthma (Bandola *et al.* 2017). Interestingly, it has been shown that NGF can mediate several functions of pDCs through signaling through p75NTR. For instance, it was shown that NGF can increase allergen-specific T cell proliferation and cytokine secretion in patients with asthma, delay the onset of autoimmune diabetes and intensified graft-versus-host disease murine models (Bandola *et al.* 2017). In this regard, it is interesting to evaluate if other functions of pDCs in mediating immune responses to infectious diseases, promoting of Tregs and induction of tolerance to oral antigens and alloantigens, neurotrophic and anti-angiogenic properties of pDCs can be regulated by NGF or other neurotrophic molecules via signaling through p75NTR or other neurotrophic factor receptors.

5.3. Cellular Regulation of Plasmacytoid Dendritic Cell Function

Considering the complexity of *in vivo* interactions of cells in tissues, understanding the cellular players that regulate diverse and sometimes opposing functions of pDCs is as crucial as dissecting the signaling pathways that can regulate pDCs. Potentially cells that may alter pDC properties might be tissue-specific and vary among different tissues. For instance, while in the cornea subbasal nerves, epithelium, stromal keratocytes, and corneal immune cells may serve as potential cellular candidates that affect pDC behavior, in the limbus, choroid, and retina, vascular endothelial cells may mainly regulate pDCs since pDCs reside in close proximity to vasculature in these tissues.

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Another important avenue is the exploration of potential crosstalk between pDCs and cellular members of the vascular system, such as vascular endothelial cells, pericytes, and vascular smooth muscle cells. As demonstrated above, pDCs in the limbus stably engulf vasculature and patrol intravascular spaces; it might be postulated that expression of certain chemokines by vasculature potently attracts pDCs and leads to special pDC-vessel arrangement observed in the limbus, choroid, and retina. It would be interesting to assess if such interactions are involved in particular pDC functions, for instance for entering blood/lymphatic system to deliver antigen to dLNs.

In summary, pDCs are the most recently identified immune cells in ocular tissues, which in addition to mediating immune response to pathogens, may contribute to several aspects of ocular tissue homeostasis including preserving ocular immune privilege, nerve maintenance and function, as well as regulating the ocular vasculature. Future studies are paramount to evaluate their biology and their role in various ocular conditions ranging from infectious or non-infectious ocular diseases such as conjunctivitis, keratitis, and uveitis to vascular diseases such as corneal neovascularization, diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Financial Support:

NIH R01-EY022695 (PH), NIH R01-EY026963 (PH), NIH R01-EY029602 (PH), NIH R21-EY025393 (PH), Eversight Eye and Vision Research Grant (PH), Massachusetts Lions Eye Research Fund Inc. (PH), Eye Bank Association of America (AJ), Research to Prevent Blindness Challenge Grant, Tufts Medical Center Institutional Support

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Highlights

- The cornea, limbus, conjunctiva, choroid, retina, and lacrimal glands are endowed with resident plasmacytoid dendritic cells.
- Corneal plasmacytoid dendritic cells secret type I interferons during herpes simplex virus-1 keratitis and limit viral propagation, dissemination to the corneal stroma, draining lymph nodes, and trigeminal ganglion. They also prevent re-programing of Tregs to effector ex-Tregs.
- Corneal plasmacytoid dendritic cells promote graft survival by inhibiting effector Th1 cells in the draining lymph nodes after corneal transplantation.
- Corneal plasmacytoid dendritic cells are pivotal for corneal nerve maintenance and function through secretion of neurotrophic molecules.
- Resident plasmacytoid dendritic cells, which accompany limbal vessels, produce anti-angiogenic molecules and contribute to corneal angiogenic privilege.

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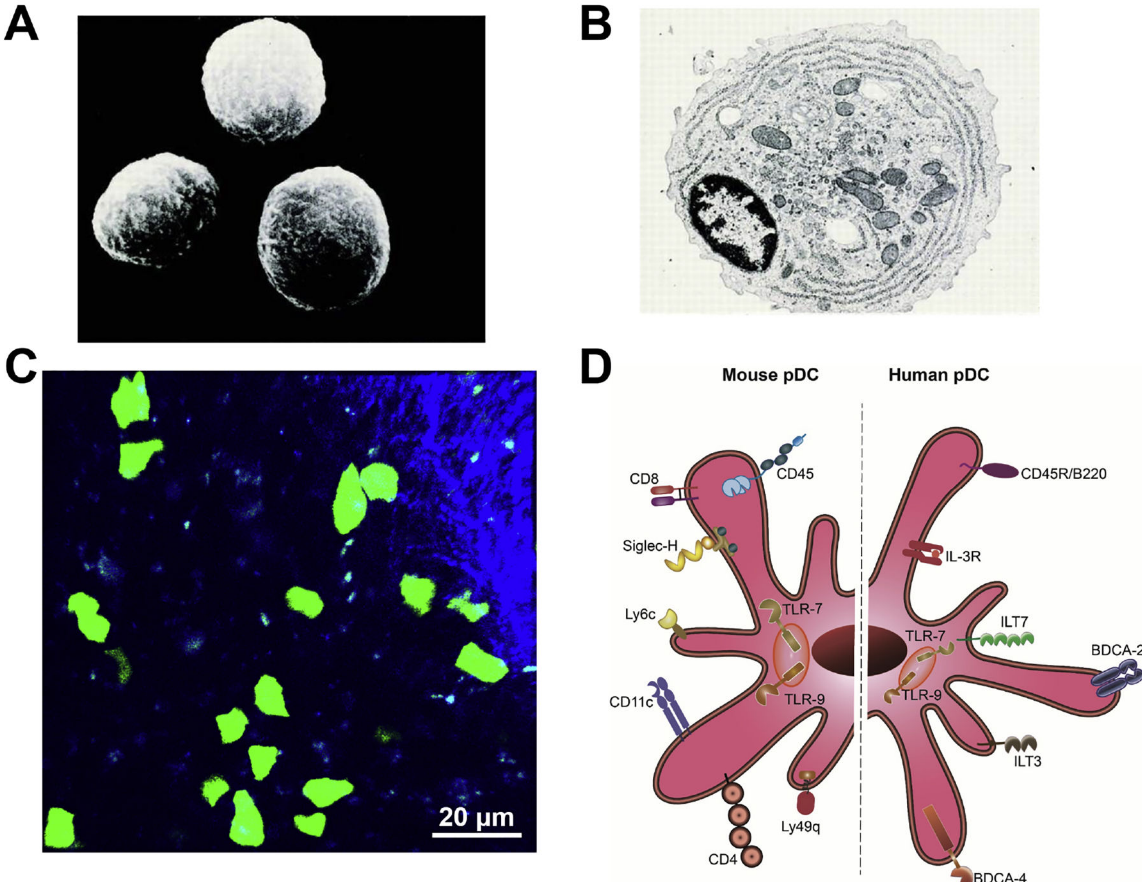

Figure 1. Illustration of plasmacytoid dendritic cells.

(**A**, **B**) Scanning electron micrograph (**A**) and transmission electron micrograph (**B**) of human pDCs isolated from peripheral blood. Magnification: ×3,500 in (**A**) and ×8,000 in (**B**); ©1997 Grouard et al. Originally published in J Exp Med. https://doi.org/10.1084/jem.185.6.1101. (**C**) Representative image of splenic pDCs in a DPE-GFP×RAG1$^{-/-}$ mouse with GFP-tagged pDCs. Scale bar: 20 μm. (**D**) Schematic representation of pDC markers in mice and humans. In humans, pDCs express the specific markers BDCA-2 and ILT-7, and share expression of BDCA-4, IL-3Rα, and ILT-3 with other immune cells. In mice, pDCs express PDCA-1, Siglec-H, CD11c, CD45R/B220, Ly6C, and Ly49Q. Both human and murine pDCs express intracellular receptors TLR-7 and TLR-9.

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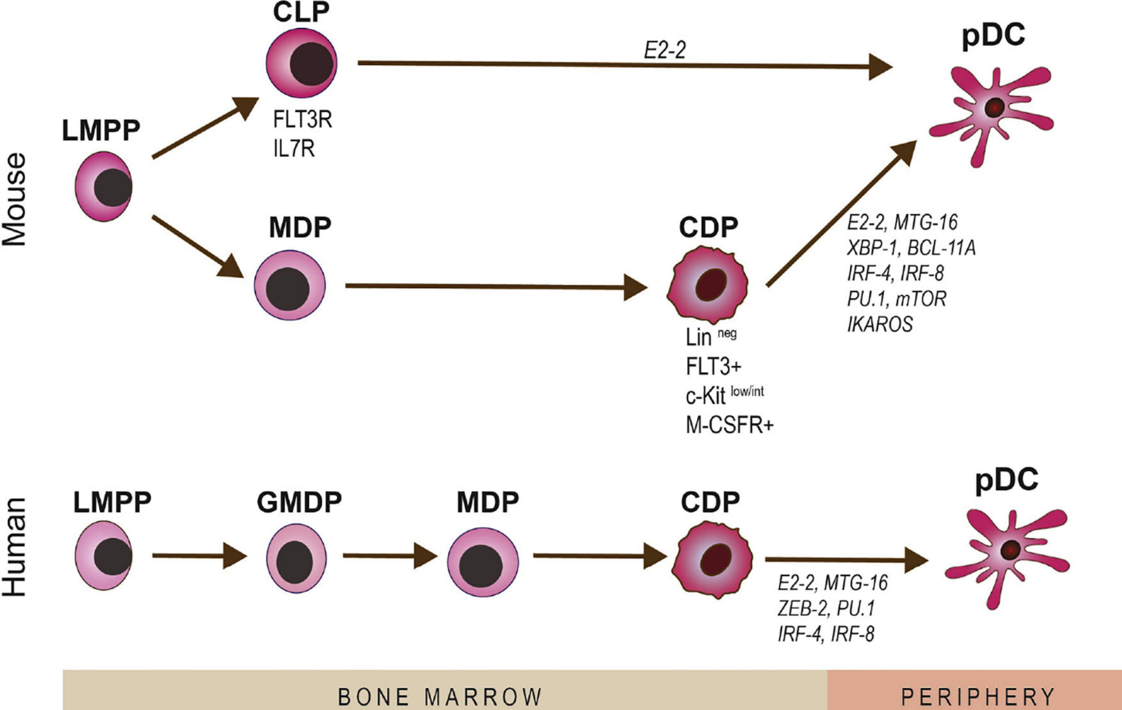

Figure 2. Schematic diagram on development of plasmacytoid dendritic cells.

As bone marrow-derived cells, pDCs can origin from both myeloid and lymphoid precursors, in mice. The cellular precursors of pDCs and transaction factors involved in development of pDCs are shown.

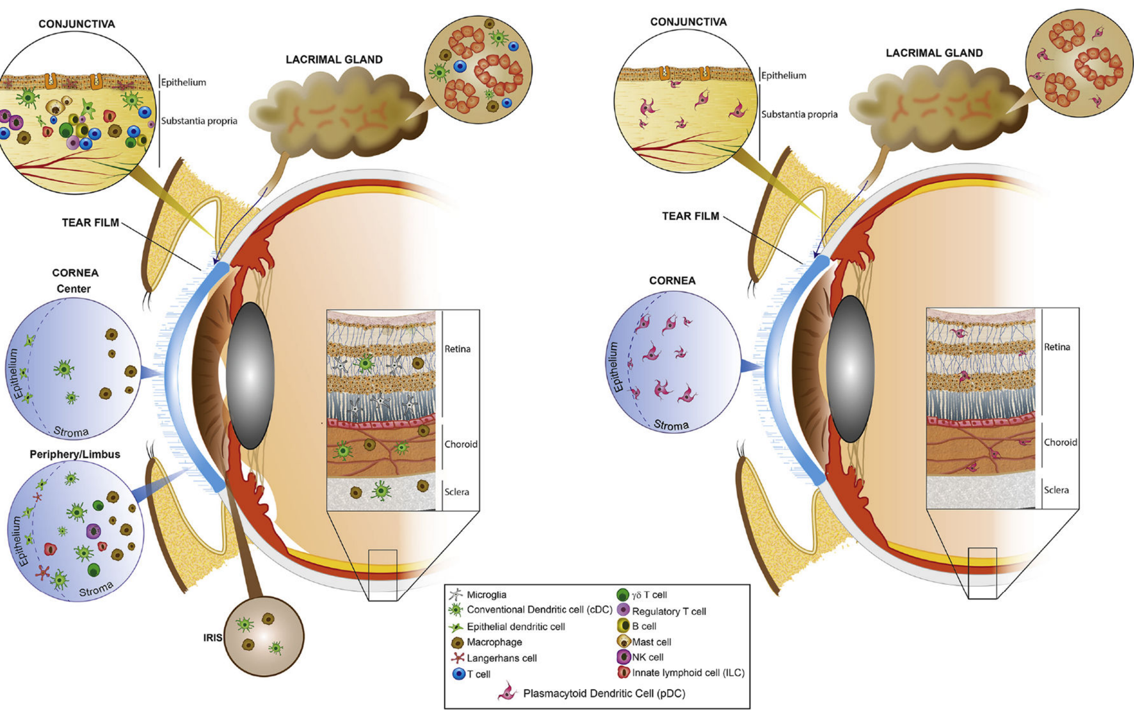

Figure 3. Schematic illustration of distribution of various resident immune cells in the ocular tissues.

(**A**) Resident immune cells are located in different parts of the ocular system. In the conjunctiva, cDCs, macrophages, B cell, and T cells are detected; in cornea, cDCs and macrophages comprise the main resident immune cells; in choroid, cDCs and macrophages and in retina microglia, perivascular macrophages, and cDCs are considered the main resident immune cells; in the lacrimal gland, cDCs, macrophages, and B cells are predominant resident immune cells. (**B**) To date, resident pDCs are reported in the central and peripheral cornea, limbus, bulbar conjunctiva, choroid, retina, and lacrimal gland

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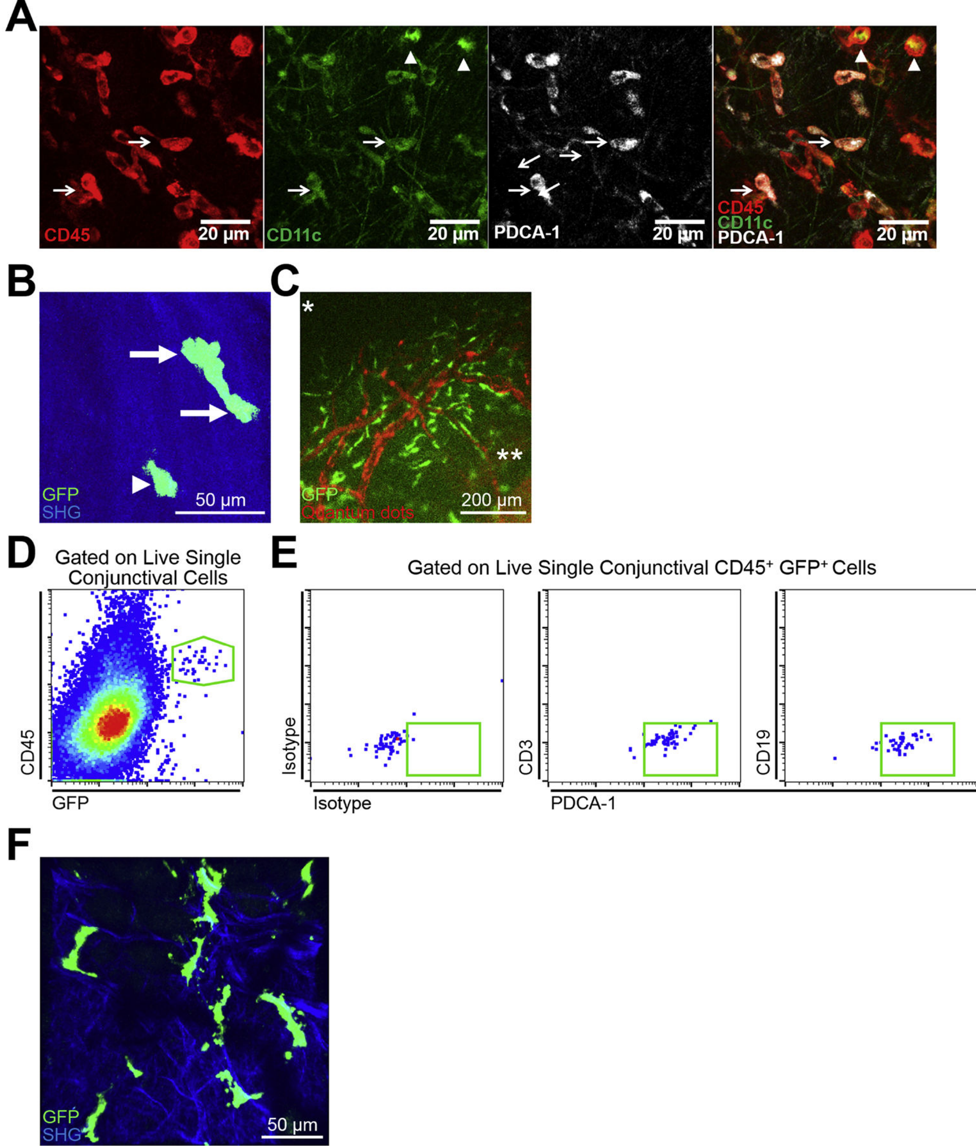

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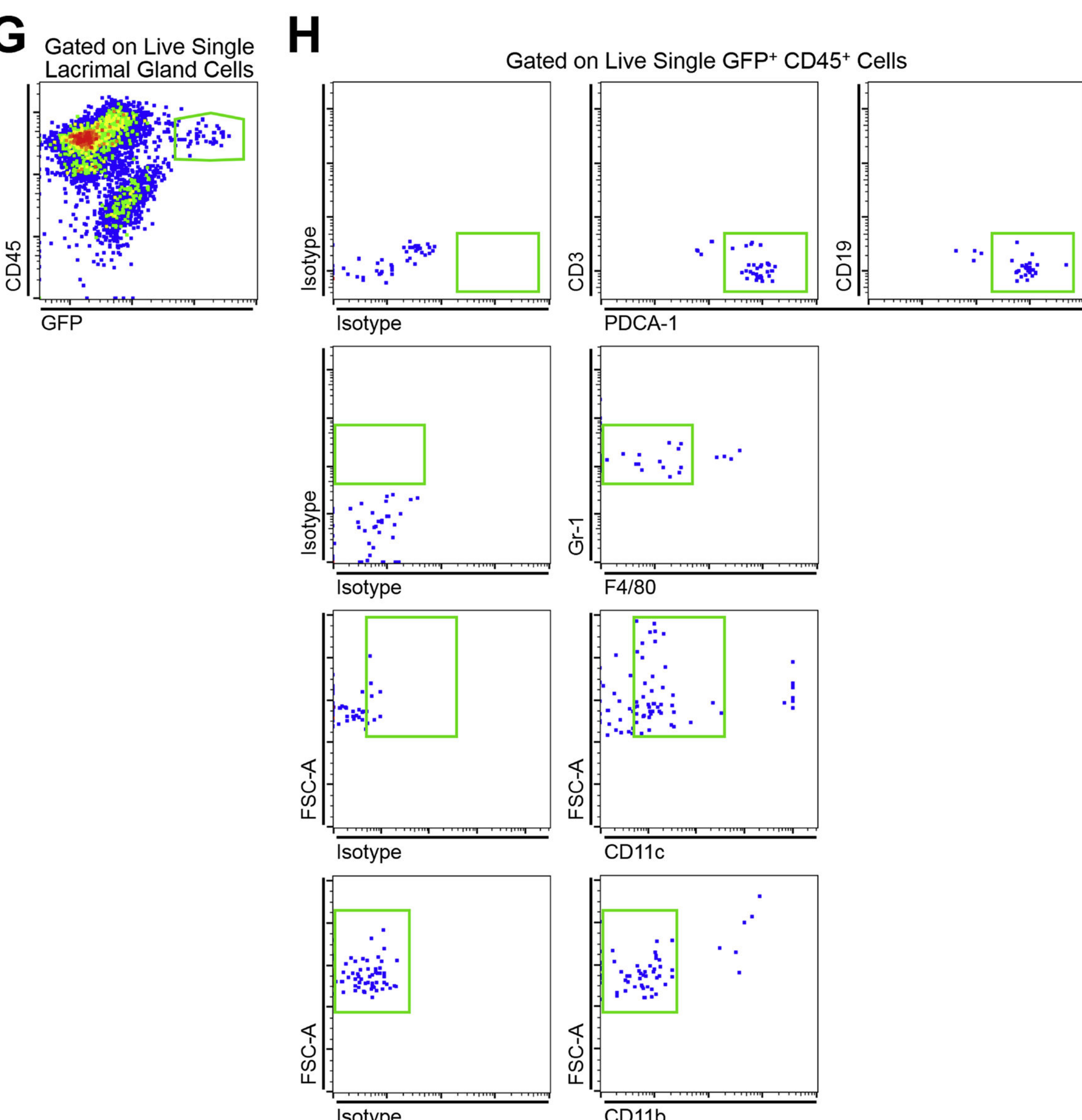

Figure 4. Presence of resident plasmacytoid dendritic cells in ocular tissues.

(**A**) Representative confocal micrographs of the limbus of a wild-type C57BL/6 mouse indicting $CD45^+$ $PDCA\text{-}1^{neg}$ $CD11c^{high}$ cDCs (arrow heads) as well as $CD45^+$ $PDCA\text{-}1^+$ $CD11c^{low}$ pDCs (arrows) during steady state. Scale bar: 20 µm. (**B**) Representative reconstructed multiphoton micrograph of cornea of a transgenic DPE-GFP×$RAG1^{-/-}$ mouse with specifically GFP-tagged plasmacytoid dendritic cells (green), highlighting typical morphology of resident corneal plasmacytoid dendritic cells with knob-like extensions (arrows) as well as less common morphology of corneal plasmacytoid dendritic cells with a round cell body without long stellates (arrow head). Second harmonic generation (SHG; blue) delineates corneal stroma. Scale bar: 50 µm. (**C**) Representative fluorescent microscopy image of the limbus in a transgenic DPE-GFP×$RAG1^{-/-}$ mouse receiving intravenous injection of quantum dots (red), reveals strategic localization of plasmacytoid dendritic cells (green) in close proximity to vessels in the limbus. Scale bar: 200 µm. (**D**)

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Representative flow cytometric dot plot on pooled conjunctiva of DPE-GFP×RAG1$^{-/-}$ mice during steady state indicating presence of CD45^{+} GFP^{+} cells among conjunctival single cells following gating out debris, dead cells, and debris (not shown). (**E**) Representative flow cytometric dot plots gated on live single CD45^{+} GFP^{+} cells of pooled conjunctiva of DPE-GFP×RAG1$^{-/-}$ mice during steady state depicting the identity of the CD45^{+} GFP^{+} cells as mainly plasmacytoid dendritic cells based on expression of PDCA-1 and lack of expression of CD3 and CD19. (**F**) Representative reconstructed multiphoton micrograph of the lacrimal gland of a transgenic DPE-GFP×RAG1$^{-/-}$ mouse, illustrating presence of resident lacrimal gland plasmacytoid dendritic cells. Second harmonic generation (SHG; blue) delineates lacrimal gland stroma. Scale bar: 50 μm. (**G**) Representative flow cytometric dot plot on lacrimal gland of a DPE-GFP×RAG1$^{-/-}$ mouse during steady state validating the presence of CD45^{+} GFP^{+} cells among lacrimal gland single cells following gating out debris, dead cells, and debris (not shown). (**H**) Representative flow cytometric dot plots gated on live single CD45^{+} GFP^{+} cells of lacrimal gland of a DPE-GFP×RAG1$^{-/-}$ mouse during steady state. Flow plots demonstrate that the majority of the CD45^{+} GFP^{+} cells are plasmacytoid dendritic cells based on expression of PDCA-1, moderate to low levels of CD11c, Gr-1 as well as lack of expression of CD3, CD19, CD11b, and F4/80; nevertheless, a minor population lack expression of PDCA-1 or express F4/80, CD11b, and/or high levels of CD11c.

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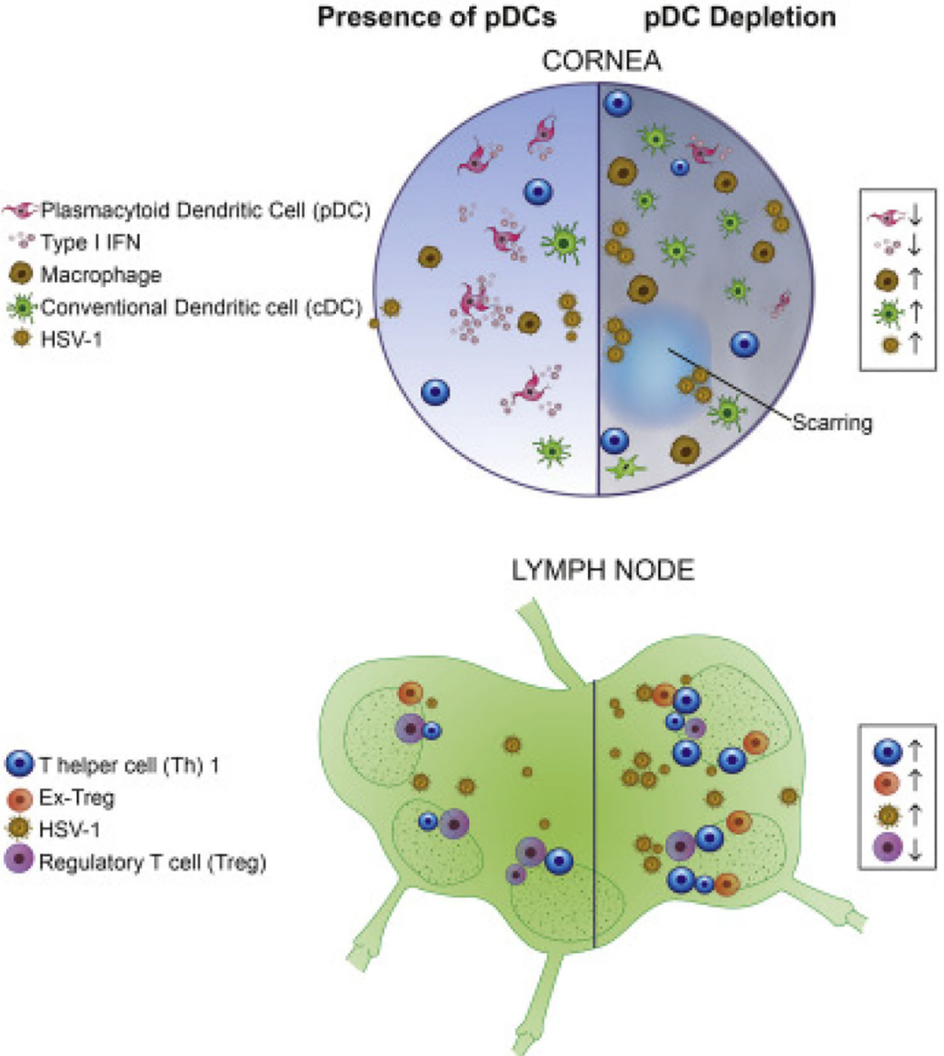

Figure 5. Schematic illustration on the role of plasmacytoid dendritic cells during herpes simplex virus-1 keratitis.

During HSV-1 keratitis, local depletion of pDCs in the cornea is accompanied by increased infiltration of cellular members of innate and adaptive immunity, including cDCs, macrophages, and ex-Tregs, enhanced viral load, and reduced IFN-α level. In the draining lymph nodes, corneal pDC depletion leads to re-programming of Tregs to effector ex-Tregs, enhanced density of Th1 cells and decreased Tregs.

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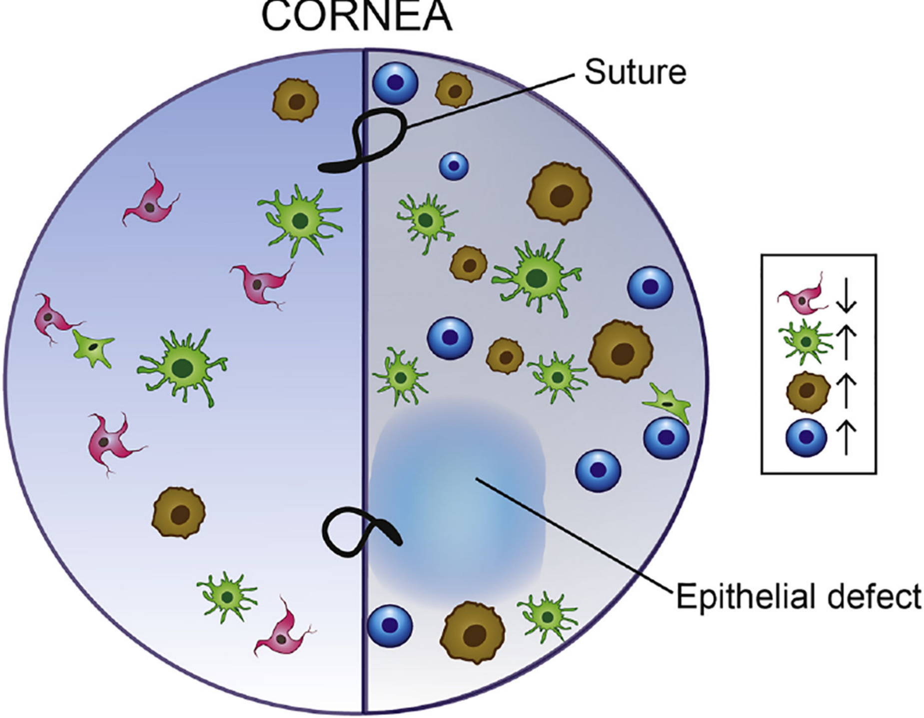

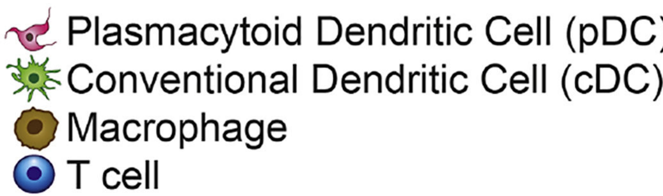

Figure 6. Schematic illustration on the role of plasmacytoid dendritic cells during sterile corneal inflammation.

During sterile corneal inflammation induced by suture placement, local depletion of pDCs leads to increased accumulation of immune cells in particular cDCs and macrophages.

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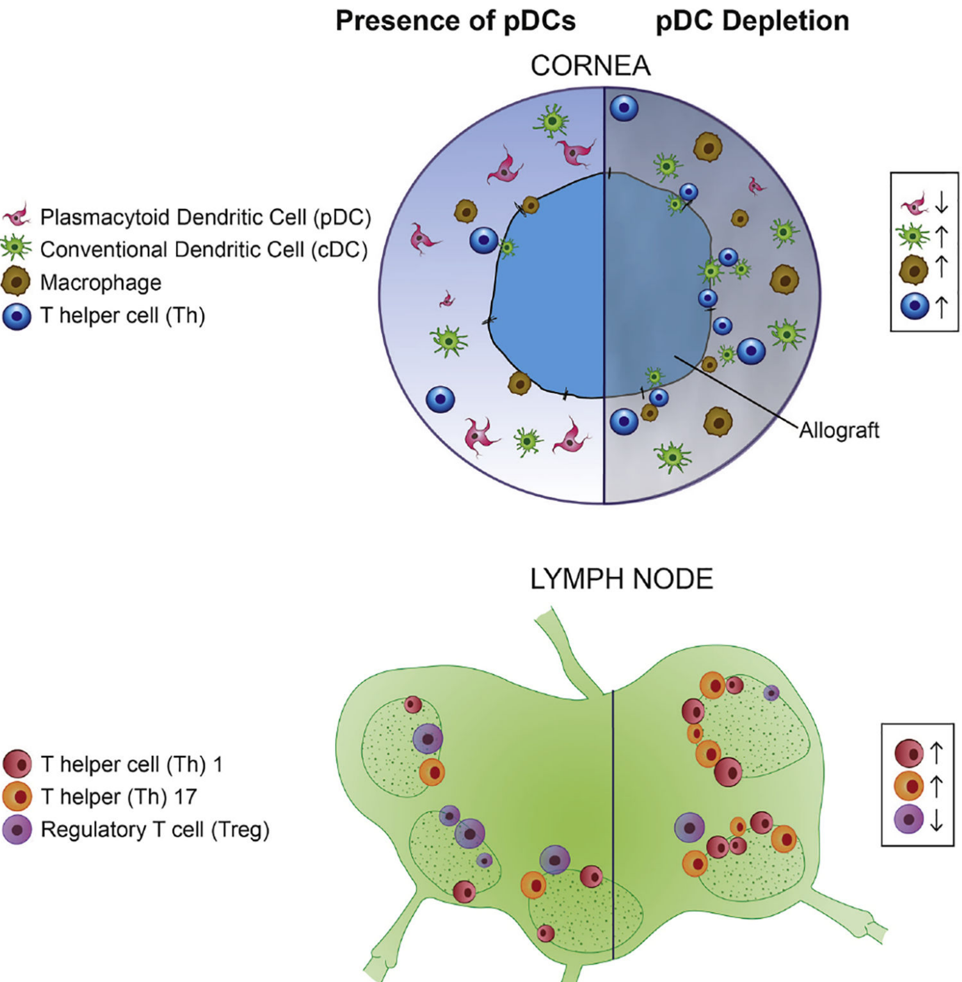

Figure 7. Schematic illustration on the role of plasmacytoid dendritic cells in corneal allograft.
During allogeneic corneal transplantation, local depletion of pDCs prior to the procedure, enhances recruitment of innate immune cells including cDCs, macrophages, and $CD4^{+}$ Th cells to the cornea and leads to enhanced generation of effector Th1 and Th17 and reduced density of Tregs in the draining lymph nodes.

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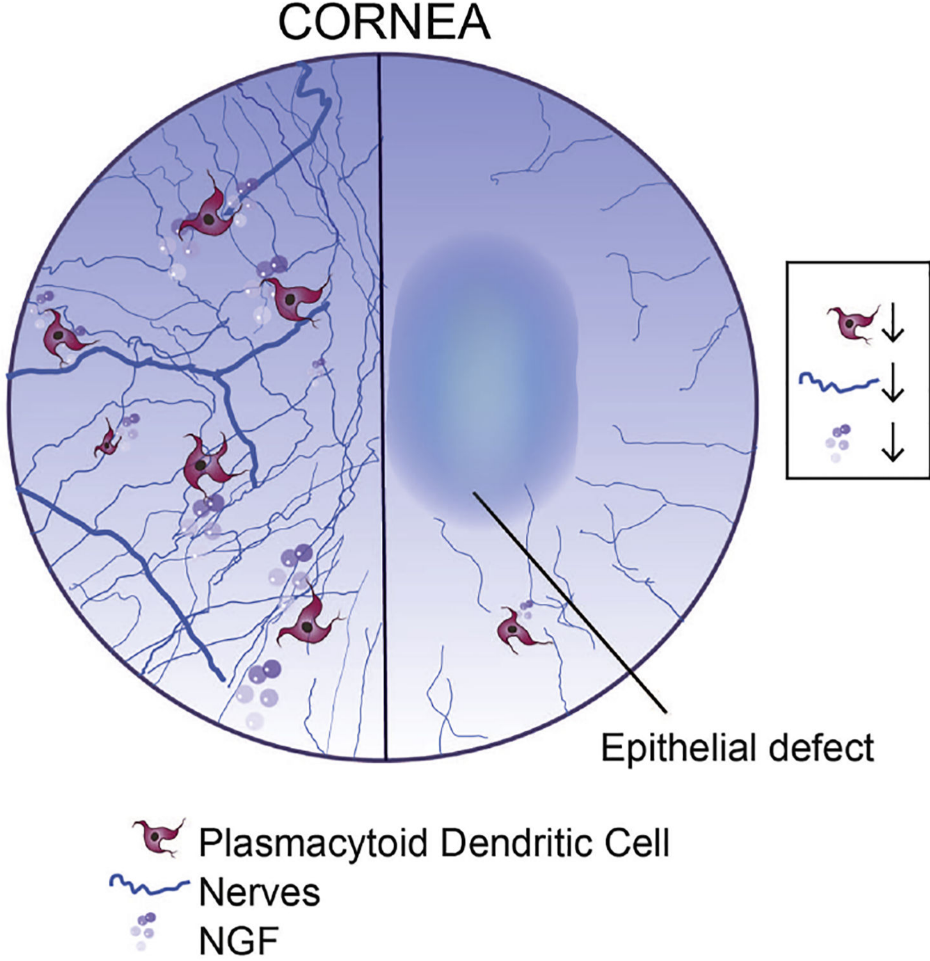

Figure 8. Schematic illustration on the role of plasmacytoid dendritic cells in homeostasis of corneal nerves.

Local depletion of pDCs in the cornea during steady state is accompanied by decreased levels of neurotrophic molecule NGF in the cornea, leading to corneal nerve degeneration, and compromise of epithelial integrity.

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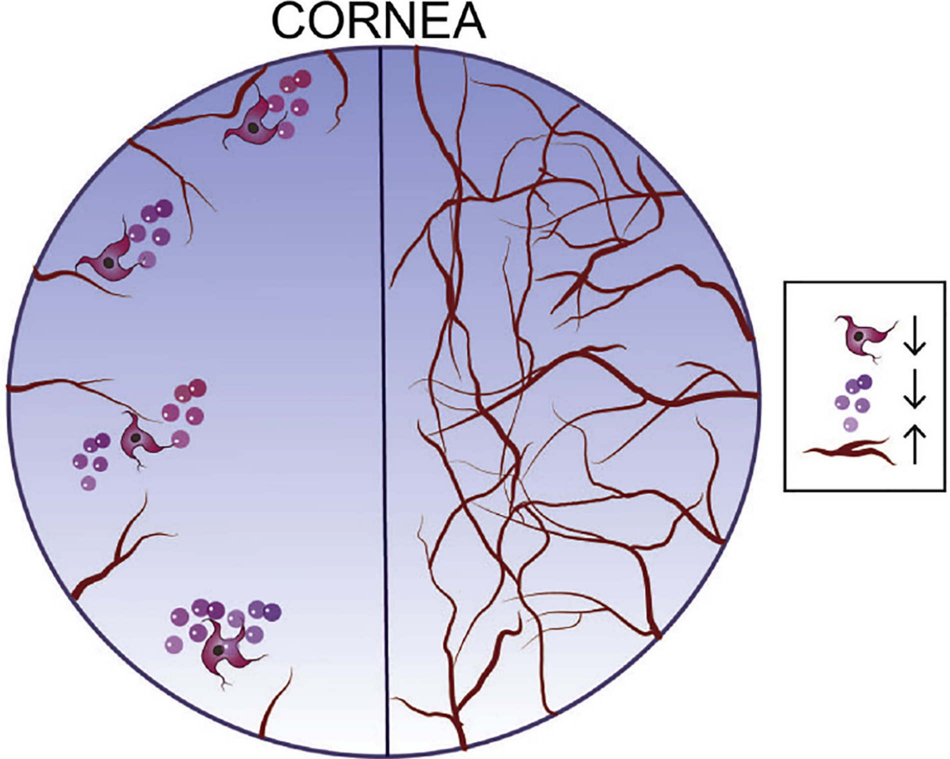

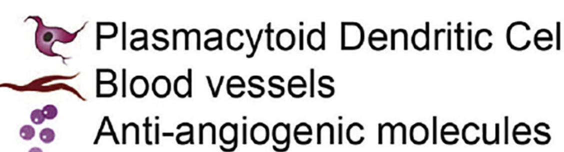

Figure 9. Schematic illustration on the role of plasmacytoid dendritic cells in corneal angiogenic privilege.

While during steady state cornea enjoys angiogenic privilege, local depletion of pDCs in the cornea is accompanied by decreased levels of anti-angiogenic molecules, leading to break down of corneal angiogenic privilege.

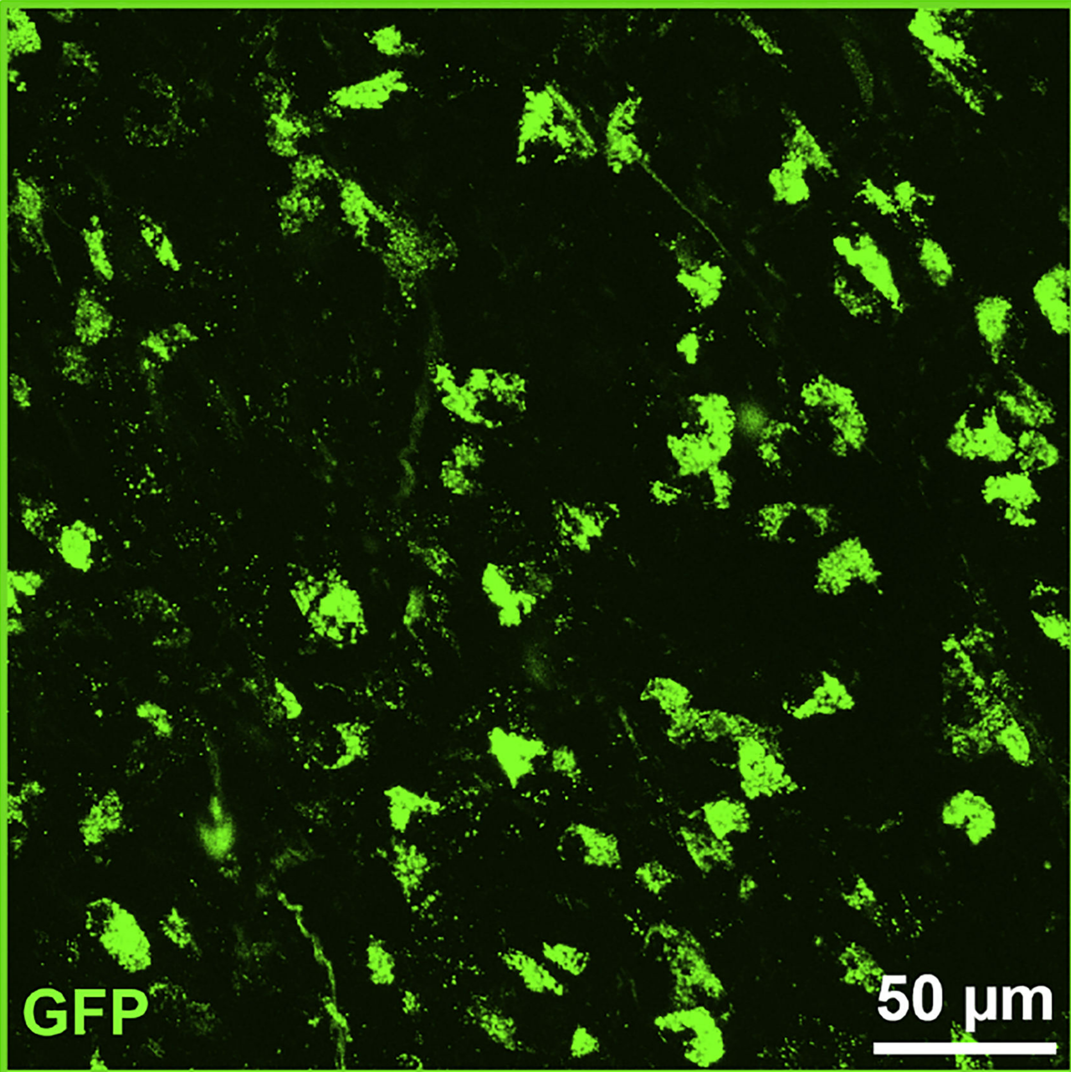

Figure 10. Representative confocal micrograph of whole-mounted cornea showing successful local adoptive transfer of plasmacytoid dendritic cells.

The figure illustrates a representative image of the paracentral cornea, 48 h following adoptive transfer of 10,000 GFP^{+} pDCs isolated from the spleen of DPE-GFP×$RAG1^{-/-}$ mouse. Scale bar: 50 μm.

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Table 1.

pDCs Markers in Humans and Mice

Marker	Human	Mouse
CD1a (Langerhans cell marker)	– (Facchetti *et al.* 1988) (Grouard *et al.* 1997) (Olweus *et al.* 1997)	N/A
CD3 (T cell marker; T cell co-receptor)	– (Muller-Hermelink *et al.* 1983) (Harris *et al.* 1987) (Horny *et al.* 1987) (Facchetti *et al.* 1988) (Grouard *et al.* 1997) (Olweus *et al.* 1997) (Bendriss-Vermare *et al.* 2001)	– (Asselin-Paturel *et al.* 2003)
CD4 (T cell co-receptor)	+ (Muller-Hermelink *et al.* 1983) (Harris *et al.* 1987) (Horny *et al.* 1987) (Facchetti *et al.* 1988) (Grouard *et al.* 1997) (Olweus *et al.* 1997) (Bendriss-Vermare *et al.* 2001)	+ (Nakano *et al.* 2001) (Martin *et al.* 2002) (Omatsu *et al.* 2005)
CD8 (T cell co-receptor)	– (Muller-Hermelink *et al.* 1983) (Harris *et al.* 1987) (Horny *et al.* 1987) (Facchetti *et al.* 1988) (Grouard *et al.* 1997) (Bendriss-Vermare *et al.* 2001)	+/– (Asselin-Paturel *et al.* 2001) (Nakano *et al.* 2001) (Martin *et al.* 2002) (Asselin-Paturel *et al.* 2003) low (Castellaneta *et al.* 2004) (Omatsu *et al.* 2005)
CD11b (αM integrin; usually non-covalently associates with β_2 integrin [CD18])	– (Harris *et al.* 1987) (Facchetti *et al.* 1988) (Grouard *et al.* 1997) (Olweus *et al.* 1997) (Bendriss-Vermare *et al.* 2001)	– (Nakano *et al.* 2001) (Martin *et al.* 2002) (Asselin-Paturel *et al.* 2003) (Castellaneta *et al.* 2004)
CD11c (α_x integrin; usually forms a heterodimer with β_2 integrin [CD18])	– (Facchetti *et al.* 1988) (Grouard *et al.* 1997) (Olweus *et al.* 1997)	+/++ (Nakano *et al.* 2001) (Asselin-Paturel *et al.* 2003) (Castellaneta *et al.* 2004) (Omatsu *et al.* 2005) (Contractor *et al.* 2007) and NK cells: (Blasius *et al.* 2007)
CD14 (LPS receptor; mainly expressed by monocytes and macrophages)	– (Facchetti *et al.* 1988) (Grouard *et al.* 1997) (Olweus *et al.* 1997) (Bendriss-Vermare *et al.* 2001)	
CD16 (low affinity IgG receptor, mainly expressed by NK cells, activated monocytes, and macrophages)	– (Grouard *et al.* 1997) (Olweus *et al.* 1997)	
CD19 (member of the Ig superfamily, expressed on all stages of B cell development from pro-B cells to mature B cells)	– (Grouard *et al.* 1997) (Olweus *et al.* 1997)	– (Nakano *et al.* 2001) (Martin *et al.* 2002) (Asselin-Paturel *et al.* 2003) (Contractor *et al.* 2007)
CD45R/B220 (an isoform of CD45, expressed at all developmental stages of B cells, from pro-B cells through mature B cells)		+ (Nakano *et al.* 2001) (Asselin-Paturel *et al.* 2003) (Castellaneta *et al.* 2004) (Omatsu *et al.* 2005) (Contractor *et al.* 2007)

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Marker	Human	Mouse
		And NK cells: (Blasius *et al.* 2007) NK cell progenitors: (Rolink *et al.* 1996)
CD123 (IL3Ra)	++ (Olweus *et al.* 1997) (Bendriss-Vermare *et al.* 2001) also present on blood monocytes: (Buelens *et al.* 2002) cDCs: (Masten *et al.* 2006) cDC precursors: (Breton *et al.* 2016), (See *et al.* 2017)	**– (As IL-3Ra)** (Martin *et al.* 2002) – (Asselin-Paturel *et al.* 2001) Low (Bjorck 2001) Low: (O'Keeffe *et al.* 2002)
CD56 (A single transmembrane glycoprotein member of the Ig superfamily, mainly expressed by NK and NKT cells)	– (Grouard *et al.* 1997) (Olweus *et al.* 1997) (Bendriss-Vermare *et al.* 2001)	
CD303 (BDCA-2; a type II transmembrane glycoprotein member of the C-type lectin superfamily)	+* (Dzionek *et al.* 2000)	N/A
CD304 (BDCA-4; a type I transmembrane protein implicated in a variety of biologic functions; VEGF165/semaphorin-3A receptor)	(Dzionek *et al.* 2000)	N/A
ILT3 (a type I membrane protein expressed by DCs and monocytes)	+ And monocytes, macrophages, and cDCs: (Cella *et al.* 1997) (Cao *et al.* 2006)	
ILT7 (a member of leukocyte immunoglobulin-like receptor family)	+* (Cao *et al.* 2006)	
Gr-1 (Ly6C/Ly6G)		+ (Nakano *et al.* 2001) low (Asselin-Paturel *et al.* 2003) low (Castellaneta *et al.* 2004) (Contractor *et al.* 2007)
Ly6C (a member of the Ly6 family of GPI linked protein, expressed by various murine immune cells)	N/A	+ (Asselin-Paturel *et al.* 2003) (Omatsu *et al.* 2005)
Ly6G (a member of the Ly6 family of GPI linked protein, expressed on the majority of myeloid cells and granulocytes)		– (Asselin-Paturel *et al.* 2003)
Ly49Q (a type II C-type lectin membrane-associated polypeptide)		+ (Omatsu *et al.* 2005)
PDCA-1 (Bst-2; a type II transmembrane glycoprotein, an IFN-induced response factor)	+ Also tumor cells: (Walter-Yohrling *et al.* 2003)	+ (Asselin-Paturel *et al.* 2003) (Blasius *et al.* 2006) (Contractor *et al.* 2007) Also other cells: (Blasius *et al.* 2006)
Siglec-H (a member of CD33-related Siglec family)	N/A	+
CD45 (a single chain type I membrane glycoprotein; expressed by all immune cells)	+ (Facchetti *et al.* 1988)	+

–: negative; +: present; ++:high levels

*:considered pDC specific

N/A: Not applicable

ILT: immunoglobulin-like transcript

BDCA: blood dendritic cell antigen

PDCA-1: plasmacytoid dendritic cell antigen-1

Bst-2: Bone marrow stromal antigen-2

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Table 2.

Distribution of pDCs in Tissues During Steady State

Tissue	Human	Mice
Secondary lymphoid organs		
Thymus	+ (Olweus *et al.* 1997 Bendriss-Vermare *et al.* 2001) (Martin-Gayo *et al.* 2010)	+ (Martin *et al.* 2002) (Asselin-Paturel *et al.* 2003) (Blasius *et al.* 2004) (Omatsu *et al.* 2005)
Spleen	+ (Velasquez-Lopera *et al.* 2008) (Boor *et al.* 2019)	+ (Asselin-Paturel *et al.* 2001) (Nakano *et al.* 2001) (Martin *et al.* 2002) (Asselin-Paturel *et al.* 2003) (Blasius *et al.* 2004) (Omatsu *et al.* 2005)
Liver	+ (Pedroza-Gonzalez *et al.* 2015)* (Boor *et al.* 2019)	+ (Blasius *et al.* 2004) (Omatsu *et al.* 2005)
Lymph nodes	+ (Olweus *et al.* 1997) (Tanis *et al.* 2004) (Boor *et al.* 2019)	+ (Nakano *et al.* 2001) (Martin *et al.* 2002) (Asselin-Paturel *et al.* 2003) (Blasius *et al.* 2004)
Tonsils	+ (Grouard *et al.* 1997) (Olweus *et al.* 1997)	N/A
Peyer's patches	+ (Jameson *et al.* 2002)	+ (Castellaneta *et al.* 2004) (Contractor *et al.* 2007)
Peripheral Tissues		
Lung	+ (Demedts *et al.* 2005) (Baharom *et al.* 2017)	+ (de Heer *et al.* 2004) (Omatsu *et al.* 2005) (Venet *et al.* 2010)
Kidney	+ (Woltman *et al.* 2007)	+ (Coates *et al.* 2004)
Vagina	N/E	+ (Lund *et al.* 2006)
Cervix	+ (Agrawal *et al.* 2009)	N/E

N/A: Not applicable

N/E: Not evaluated

*In patients with hepatic tumors, in sample distant from tumor (at least 1 cm)