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Models of dendritic cell development correlate ontogeny with function

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Abstract

Rapid advances have been made to uncover the mechanisms that regulate dendritic cell (DC) development, and in turn, how models of development can be employed to define dendritic cell function. Models of DC development have been used to define the unique functions of DC subsets during immune responses to distinct pathogens. More recently, models of DC function have expanded to include their homeostatic and inflammatory physiology, modes of communication with various innate and adaptive immune lineages, and specialized functions across different lymphoid organs. New models of DC development call for revisions of previously accepted paradigms with respect to the ontogeny of plasmacytoid DC (pDC) and classical DC (cDC) subsets. By far, development of the cDC1 subset is best understood, and models have now been developed that can separate deficiencies in development from deficiencies in function. Such models are lacking for pDCs and cDC2s, limiting the depth of our understanding of their unique and essential roles during immune responses. If novel immunotherapies aim to harness the functions of human DCs, understanding of DC development will be essential to develop models DC function. Here we review emerging models of DC development and function.

1. Introduction

Hematopoiesis is central to models of dendritic cell (DC) development. An early stage of fate determination is the divergence of hematopoietic stem cells (HSC) into multipotent progenitors (MPPs) restricted to myeloid and lymphoid lineages (Laurenti & Gottgens, 2018). DCs are widely accepted as constituents of the myeloid lineage and several transcription factors have been identified to regulate specification of DC subsets (Murphy et al., 2016). High-dimensional, single-cell analyses have permitted precise characterization of phenotypic heterogeneity of cellular populations in the bone marrow. For example, what was once considered a single progenitor, the common dendritic cell progenitor (CDP) (Naik et al., 2007), is now understood to be heterogeneous with discrete fate potentials (Bagadia et al., 2019). This enhanced resolution has allowed us to reexamine the mechanisms by which known regulators of DC development control fate decisions both spatially and temporally

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(Bagadia, Huang, Liu, & Murphy, 2019). Here we highlight advances in our understanding of DC development and the models currently used to determine their function.

2. Marker-based identification of murine DC progenitors

The markers used to identify multipotent mouse and human DC progenitors have been reviewed recently (Anderson, Murphy, & Briseno, 2017; Bagadia, Huang, Liu, & Murphy, 2019; Murphy et al., 2016). In mice, clonogenic progenitors of cDC subsets were identified independently by two groups. In the first, singe cell RNA-Seq was used to identify transcriptional heterogeneity within previously defined MDPs, CDPs, and pre-cDCs (Schlitzer et al., 2015). Differential expression of genes encoding surface markers informed analysis of marker heterogeneity by flow cytometry, which revealed novel clonogenic progenitors within the previously defined pre-cDC. Progenitors specified to the cDC1 and cDC2 subsets were defined as Lin-CD11c^{+}-MHCII^{-}CD135^{+}CD172α^{-}, and SiglecH^{-}Ly6C^{-} or SiglecH^{-}Ly6C^{+}, respectively. In the second study, *Zbtb46*-GFP expression marked cells committed to the cDC lineage in the bone marrow, and revealed population specified to cDC1 and cDC2 subsets (Grajales-Reyes et al., 2015). The cDC1-restricted progenitor, referred to as the pre-cDC1, can be identified in the bone marrow as Lin^{-}Zbtb46^{GFP+}CD117^{int}CD11c^{+}MHCII^{int}. Alternatively, the Lin^{-}Zbtb46^{GFP+}CD117^{-}CD11c^{+}MHCII^{-} population in the bone marrow is restricted to the cDC2 subset, and is referred to as the precDC2. Recently, a progenitor within the CDP was identified on the basis of *Zbtb46, Id2*, and *Nfil3* expression (Bagadia, Huang, Liu, Durai, et al., 2019). Genetic analysis revealed that, like the pre-cDC1, specification within the CDP is dependent on *Irf8* and *Nfil3* (Bagadia, Huang, Liu, Durai, et al., 2019; Durai et al., 2019). Although lacking cDC1s in vivo, a *Zbtb46*$^{GFP+}$ progenitor is present within the CDP of *Id2*-deficient bone marrow. These observations suggest that specification to the cDC1 subset occurs as early as the CDP.

3. Myeloid and lymphoid origins of DC subsets

Deficiency in PU.1 results in multi-lineage defects during embryonic hematopoiesis and results in embryonic lethality (Scott et al., 1997; Scott, Simon, Anastasi, & Singh, 1994). However, by ectopically modulating levels of PU.1 expression, it has been shown that high levels of PU.1 can drive the divergence of multipotent progenitors (MMPs) to the myeloid lineage (DeKoter, Lee, & Singh, 2002). The mechanism by which PU.1 expression levels are controlled in MPPs remains an active area of research. Some groups have proposed that PU.1 enforces lineage divergences through a mechanism of transcriptional autoactivation, which maintains high PU.1 expression (Leddin et al., 2011). Others have provided evidence for a feed forward mechanism between the cell cycle length and PU.1 protein levels (Kueh, Champhekar, Nutt, Elowitz, & Rothenberg, 2013). However, it is not presently possible to control the length of the cell cycle as an independent variable, so its role in fate restriction remains unclear. With respect to DC development, PU.1-deficient bone marrow progenitors fail to express Flt3 and do not generate mature DC subsets (Anderson et al., 2000; Carotta et al., 2010). In addition, PU.1 supports IRF8 function through direct interaction, which regulates cDC1 development and pDC function (Anderson et al., 2000; Tailor, Tamura, Morse, & Ozato, 2008).

Early studies of *Irf8*-deficient mice and human myeloid leukemias with reduced *Irf8* expression revealed its impact on myeloid cell homeostasis (Holtschke et al., 1996; Schmidt et al., 1998). It was subsequently shown that *Irf8* is necessary for the development of cDC1s and pDCs (Aliberti et al., 2003; Schiavoni et al., 2004, 2002). Competitive chimeras of $Irf8^{-/-}$ and $Irf8^{+/+}$ bone marrow revealed that $Irf8^{-/-}$ CDPs and all DC subsets have reduced frequencies in vivo but DC2s appear to develop normally in $Irf8^{-/-}$ mice (Becker et al., 2012). Deficiency in Runx1 and its binding partner Cbfβ leads to increased numbers of granulocyte and monocyte progenitors (GMP), loss of $Flt3^{+}$ MDPs, development of myeloproliferative disorders, and absence of DC populations (Becker et al., 2012; Satpathy et al., 2014). Forced expression of *Irf8* in *Cbfβ*-deficient bone marrow in vitro rescued defects in DC development, suggesting that the *Runx1*:*Cbfβ* axis regulates DC development through the induction of *Irf8* expression. It has not been established whether these factors act directly on the *Irf8* locus or influence *Irf8* expression indirectly during DC development.

Major subsets of immune cells of the hematopoietic system can be divided conceptually into two major lineages—myeloid and lymphoid. This distinction is derived from observations that multipotent progenitors (MPP) can give rise to a common lymphoid progenitor (CLP) with restriction to the lymphoid lineage and a common myeloid progenitor (CMP) with restriction to the myeloid lineage (Akashi, Traver, Miyamoto, & Weissman, 2000; Kondo, Weissman, & Akashi, 1997). More recently, subsets of MPPs with developmental potentials skewed to lymphoid or myeloid lineages have been identified (Pietras et al., 2015; Yamamoto et al., 2013). Therefore, the stage at which a myeloid-lymphoid divergence occurs remains an active area of investigation.

The ontogeny of pDCs has challenged models of early lymphoid-myeloid divergence. They were originally considered to have myeloid origins and be ontogenetically related to classical dendritic cells (Cella et al., 1999; Grouard et al., 1997; Olweus et al., 1997). Contrary to models that propose a myeloid origin of pDCs, thymocytes cultured in vitro with Flt3l give rise to pDCs while also maintaining lymphoid characteristics (Corcoran et al., 2003; D'Amico & Wu, 2003; Sathe, Vremec, Wu, Corcoran, & Shortman, 2013; Shigematsu et al., 2004). IL7r is commonly used to define progenitors restricted to lymphoid lineages. Therefore, a mouse model with CRE-recombinase driven by the endogenous *Il7r* locus and loxp-STOP-loxp-YFP-reporter at the *Rosa26* locus was generated to study the lymphoid origins of immune cells in vivo (Schlenner et al., 2010). Analysis of all major myeloid and lymphoid subsets revealed that a majority of pDCs, but not cDCs, in the thymus and spleen were marked by YFP expression. It is also widely accepted that pDCs can be derived from myeloid-restricted progenitors that do not express IL7r during their development, such as MDPs and CDPs (Auffray et al., 2009; Fogg et al., 2006). It has also been reported that the $Lin^{-}CD117^{int/lo}CD135^{+}CD115^{-}$ fraction of the bone marrow is enriched in progenitors of pDCs when compared to CDPs, which are $CD115^{+}$ (Onai et al., 2013, 2007). However, IL7r is the only marker that separates this pDC-enriched population from CLPs (Kondo et al., 1997). Near these dimensions of surface marker expression, it was recently reported that $Lin^{-}CD16/32^{-}B220^{-}Ly6C^{-}IL7r^{+}CD117^{int/lo}CD135^{+}Ly6D^{+}SiglecH^{+}$ progenitors give rise exclusively to pDCs (Rodrigues et al., 2018). Single cell and bulk transcriptome analyses revealed differences between pDCs derived from CDPs and CLPs. The former express genes conventionally associated with lymphoid and the latter with myeloid lineages. *Zbtb46* is

expressed by cDCs and can be used to identify progenitors and tissue resident cells that belong to the cDC lineage (Anderson et al., 2017; Bagadia, Huang, Liu, Durai, et al., 2019; Grajales-Reyes et al., 2015; Meredith et al., 2012; Rodrigues et al., 2018; Satpathy et al., 2012; Wu et al., 2016). Splenic pDCs that coexpressed *Zbtb46* with the pDC marker, BST2, were classified as cDC-like pDCs (Rodrigues et al., 2018). A mechanism that can explain the differential ontogeny of lymphoid and myeloid pDCs has not been defined. However, functions classically ascribed to cDCs were observed for the splenic lymphoid subset of pDC (Rodrigues et al., 2018). Pre-cDC2s are transcriptionally similar to the proposed lymphoid pDC, including for the expression of the proposed lineage marker, *Bst* (Grajales-Reyes et al., 2015). Until a unique regulator of lymphoid pDC development is identified, it will not be possible to genetically identify an in vivo requirement for this subset.

4. Mechanisms of dendritic cell specification

The pre-cDC, defined as $Lin^{-}CD135^{+}CD11c^{+}MHCII^{-}CD172^{-}CX3CR1\text{-}GFP^{+}$, was originally reported to give rise to cDC1s and cDC2s but not pDCs in mice, and a similar population was described in humans (Breton, Lee, Liu, & Nussenzweig, 2015; Breton et al., 2015, 2016; Liu et al., 2009). However, it is now understood that substantial heterogeneity exists within this population. The studies that identified this heterogeneity were able to isolate clonogenic cDC progenitors on the basis of CD115, CD117, SiglecH, Ly6C, Zbtb46, and CCR9 expression (Breton et al., 2016; Grajales-Reyes et al., 2015; Schlitzer et al., 2011, 2015; See et al., 2017; Villani et al., 2017).

A mechanistic explanation for the exclusion of pDC potential is the induction of ID2 to inhibit E2–2, which is necessary for pDC development (Reizis, Bunin, Ghosh, Lewis, & Sisirak, 2011). E-proteins, including E2–2/*Tcf4*, E2A/*Tcf3* and HEB/*Tcf12* are basic helix-loop-helix (HLH) domain-containing transcription factors that activate transcription when heterodimerized with other HLH transcription factors, such as MyoD (Lassar et al., 1989; Murre et al., 1989; Wang & Baker, 2015). ID proteins are HLH domain-containing proteins that lack a basic DNA-binding domain. Therefore, ID proteins can heterodimerize with bHLH proteins to inhibit E-protein-mediated transcription (Benezra, Davis, Lockshon, Turner, & Weintraub, 1990). Independent studies reported that *Id2* and *Tcf4* are required for the development of cDC1s and pDCs, respectively (Cisse et al., 2008; Hacker et al., 2003). The subsequent identification of the pre-cDC as a progenitor with bipotential for cDC1 and cDC2 subsets led to the hypothesis that repression of E2.2 by ID2 is necessary for cDC specification (Geissmann et al., 2010; Liu et al., 2009). However, evidence that cDC2s develop normally in $Id2^{-/-}$ mice is inconsistent with this model because it would also require a dependence on Id2 for cDC2 development.

Tcf4 is expressed by all DC subsets but is expressed most highly in pDCs. A more precise analysis of *Tcf4* isoform expression by RNA-Seq revealed two isoforms, with the long isoform being expressed exclusively by pDCs (Grajkowska et al., 2017). ATAC-Seq of pDCs and cDCs revealed that chromatin around the promoter of the *Tcf4* is accessible in all DC subsets and that specificity of *Tcf4* expression is controlled by a 3′ enhancer accessible only in pDCs. Deletion of the long-isoform of *Tcf4* was not sufficient to ablate

pDC development. However, it was proposed that early expression of the long-isoform in the CDP could be an event that leads to higher TCF4 expression and commitment to the pDC lineage (Grajkowska et al., 2017; Reizis, 2019).

Mounting evidence supports a model of epistasis between ZEB2 and ID2 that controls specification of bone marrow progenitors to the pDC and cDC1 subsets, respectively (Bagadia, Huang, Liu, & Murphy, 2019). Two independent groups reported that the transcription factor, ZEB2, is also required for pDC development (Scott et al., 2018; Wu et al., 2016). Although the mechanism by which ZEB2 regulates DC development remains an active area of research, both studies provided preliminary evidence of epistasis with ID2. They both reported that DC2s deficient in *Zeb2* in vivo have an increase in *Id2* expression. In silico motif analysis of the *Id2* promoter identified a putative ZEB2 binding site by ChIP-qPCR. It was thus proposed that ZEB2 acts to directly inhibit transcription of *Id2* (Scott et al., 2018; Wu, Briseno, Grajales-Reyes, et al., 2016). However, high throughput ChIP-Seq was not conducted and a ZEB2 antibody is not publicly available. Therefore, a requirement for ZEB2 in the direct repression of *Id2* transcription remains to be established.

A distinct population of pre-cDC1s was recently identified by the expression of *Zbtb46* within the CDP. Results indicated that specification of pre-cDC1s occurs earlier than the acquisition of CD11c and MHCII, and before the loss of CD115 expression (Bagadia, Huang, Liu, Durai, et al., 2019). Compared to CDPs, this earlier progenitor expressed higher levels of transcription factors that regulate cDC1 development, including *Nfil3* and *Batf3*. Likewise, *Zeb2* repression coincided with *Id2* induction. Consistent with these results, *Zbtb46* induction as a marker of specification did not occur in $Nfil3^{-/-}$. Loss of cDC1s in $Nfil3^{-/-}$ mice was rescued in $Zeb2^{-/-}Nfil3^{-/-}$ mice, demonstrating that *Nfil3* is upstream of *Zeb2* with respect to cDC1 development, possibly as a repressor of *Zeb2* expression. Similarly, loss of cDC1s in $Id2^{-/-}$ mice could be rescued in $Id2^{-/-}Zeb2^{-/-}$ mice, demonstrating that *Zeb2* acts downstream of *Id2* with respect to cDC1 development, possibly as an indirect repressor of *Zeb2* expression. However, *Id2* was expressed at normal levels in $Zeb2^{-/-}Nfil3^{-/-}$ despite a requirement for *Nfil3* for cDC1 specification. This suggests that *Zeb2* acts upstream of *Id2*, possibly by repressing *Id2* expression. This would establish a mutually repressive epistasis between *Zeb2* and *Id2* that could be the basis for exclusion of pDC fate. *Zeb2* expression does not mark pDC-specified progenitors within the CDP and is not required for cDC2 development. Therefore, mutual repression of *Zeb2* and *Id2* is not sufficient to exclude cDC2 specification alone. Together these data suggest that pDCs and cDC1s share a common progenitor that diverges from the CDP prior to cDC2 specification (Bagadia, Huang, Liu, & Murphy, 2019).

Maintenance of *Irf8* expression is required for cDC1 specification and survival in peripheral tissues (Aliberti et al., 2003; Schiavoni et al., 2004, 2002). Development of pDCs was also thought to be dependent on *Irf8* (Schiavoni et al., 2002). However, it was recently shown that pDCs develop in $Irf8^{-/-}$ mice but have an altered surface phenotype (Sichien et al., 2016). *Irf8*, however, was required for pDC function. It was observed that $Irf8^{-/-}$ pDCs induced the expression of *Irf4*, and it was reported that pDC development could be abrogated in $Irf8^{-/-}Irf4^{-/-}$. Therefore, *Irf4* can rescue pDC development, and compensate for loss of *Irf8*.

However, *Irf8*$^{-/-}$ *Irf4*$^{-/-}$ mice lack development of all DC lineages, so the mechanism by which *Irf4* is sufficient to compensate for Irf8 remains unclear (Tamura et al., 2005).

5. Models of cDC1 development inform function

The discovery that *Batf3*$^{-/-}$ mice lack cDC1s provided a model to demonstrate cDC1 function. *Batf3*-deficient mice are susceptible to certain viral infections and rejection of immunogenic tumors (Hildner et al., 2008). This model has been used to further demonstrate that cDC1s are essential during the acute, innate phase of some infections. For example, cDC1s express high levels of *Tlr11* (Merad, Sathe, Helft, Miller, & Mortha, 2013), which detects a *Toxoplasma gondii* antigen and induces IL-12 production (Yarovinsky et al., 2005). This suggests that cDC1s are poised to respond to *Toxoplama gondii* infection. Indeed, *Batf3*-dependent DCs were demonstrated to be an early source of IL-12 that is required to prevent mortality to infection by this parasite (Mashayekhi et al., 2011). *Batf3*$^{-/-}$ also helped define the shared ontogeny of cDC1 subsets across tissues independent of variable surface marker expression (Edelson et al., 2011, 2010).

Interest in cDC1 function continues as they are essential for tumor rejection and mediate responses to cancer immunotherapy. Although previous studies revealed the importance cDC1 in immunogenic tumor rejection (Hildner et al., 2008), it remained unknown whether immunotherapy-mediated rejection of non-immunogenic tumors would also be dependent on cDC1s. To that end, it was recently shown using a non-immunogenic sarcoma cell line that therapeutic responses to checkpoint blockade (anti-CTLA4 and/or anti-PD-1) are abrogated in *Batf3*$^{-/-}$ mice (Gubin et al., 2014). These results are consistent with reports that cDC1s promote rejection after immunotherapy against oncogene-driven tumors (Salmon et al., 2016). Tumor infiltration by antigen-specific effector T cells is also dependent on cDC1s (Spranger, Dai, Horton, & Gajewski, 2017). Therefore, mounting evidence suggests that therapeutic modulation of DC function in vivo could alter the efficacy of cancer immunotherapies. However, the genes essential for cDC1-mediated rejection remain elusive, hindering the identification of molecular targets for novel immunotherapy (Theisen et al., 2019).

Cross presentation of tumor antigens to CD8 T cells is a process that has been widely studied to identify novel molecular targets of DC1 function. As reviewed recently, the molecular mechanisms of cross presentation have largely used models of bone marrow or monocyte-derived DCs cultured in GM-CSF or a combination of GM-CSF and IL-4 (Helft et al., 2015; Theisen & Murphy, 2017). These models are problematic because DC development in vivo is not dependent on these cytokines, the in vivo counterparts of GM-CSF-derived DCs are not clear, and the cells produced are heterogeneous populations of myeloid cells (Edelson et al., 2011; Helft et al., 2015).

Although numerous studies suggest that monocyte derived DCs have essential functions in vivo (Greter et al., 2012; King, Kroenke, & Segal, 2010; Nakano et al., 2009; Plantinga et al., 2013; Tamoutounour et al., 2013), their existence and ontogenetic relationship to cDCs remains an active area of debate (Wu, Briseno, Durai, et al., 2016). With respect to development, for example, the ontogeny of cross-presenting monocyte-derived DCs in

vitro is distinct from in vivo cDC1s, since their development is *Batf3*-independent but *Irf4*-dependent (Briseno et al., 2016). Alternatively, DCs derived from in vitro cultures of bone marrow supplemented with Flt3l are widely accepted to better recapitulate the development and function of in vivo DC subsets (Mayer et al., 2014; Naik et al., 2005). Injection of FLT3L, but not GM-CSF or IL-4, into mice selectively expands dendritic cells, and this phenomenon also occurs in humans (Maraskovsky et al., 1996; Waskow et al., 2008). Although FLT3L has the most potent effect on the expansion of DC progenitors in vivo and DC development is significantly impaired in *Flt3l*$^{-/-}$ and *Flt3*$^{-/-}$ mice, it has been observed that residual populations of DCs are present and increase with age (Durai et al., 2018; Ginhoux et al., 2009; McKenna et al., 2000). Recently it was demonstrated that the cytokines SCF and M-CSF can compensate for deficiency in FLT3L signaling and support DC development (Durai et al., 2018).

An in-depth review of antigen processing and cross-presentation by cDC1s is beyond the scope of this review (Theisen & Murphy, 2017), but a few studies will be noted here to illustrate discrepancies in functional studies that use GM-CSF-derived, Flt3l-derived, and in vivo DC models. Rab43 is highly expressed in dendritic cells and was recently identified as a regulator of antigen cross presentation by cDC1s ex vivo and in FLT3L-derived but not GM-CSF-derived DC cultures (Kretzer et al., 2016). Experiments that knocked down of Sec22b expression by shRNA in GM-CSF-derived DCs suggested that it is necessary for phagosome maturation, antigen degradation, and cross-presentation (Cebrian et al., 2011). Recently, two independent studies using *Itgax*-Cre *Sec22b*$^{fl/fl}$ mice reported conflicting results with respect to Sec22-dependent cDC1 functions. One study reported defects in cDC1-mediated CD8 T cell activation and inhibition of tumor growth (Alloatti et al., 2017). This was in contrast to another study that reported no defects in antigen presentation by standard in vivo methods (Wu et al., 2017). The activity of transcription factors, such as CIITA, in vivo and FLT3L cultures has also been shown to be distinct from models that have used GM-CSF-derived DCs (Anderson, Grajales-Reyes, et al., 2017). These examples highlight the need to revisit the relevance of discoveries made using GM-CSF-derived DCs.

Contemporary models of cDC1 function should use ontogenetically relevant methods of generating in vitro counterparts of cDC1s. Such an approach was used recently to screen for regulators of antigen cross presentation in cDC1s. Numerous candidate genes were identified and screened in vitro by CRISPR-Cas9-mediated target deletion (Theisen et al., 2018). DCs were generated from FLT3L cultures, and cDC1s were sorted to determine the impact of specific genetic mutations on antigen cross presentation to CD8 T cells. *Wdfy4* was revealed to be required for cross-presentation by cDC1s. In turn, *Wdfy4*$^{-/-}$ were generated and used to demonstrate that *Wdfy4* is required for cross presentation by cDC1s in vivo. cDC1s developed normally in these mice but failed to reject immunogenic tumors, were susceptible to infection with intracellular parasites, and failed to prime CD8 T cells to viral infections (Theisen et al., 2018). *Wdfy4*$^{-/-}$ will be a useful model to dissect defects in cross presentation from other cDC1 functions, which to date have been identified using models with broad developmental defects.

6. pDC function is still a black box

Plasmacytoid DCs are major producers of type I interferons during viral infections (Reizis, Colonna, Trinchieri, Barrat, & Gilliet, 2011). They are poised to respond to viral antigens through the steady state expression of *Tlr7* and *Tlr9* (Asselin-Paturel & Trinchieri, 2005). Ablation of pDC populations revealed that they are required to limit viral burden after infection with murine cytomegalovirus, and pDCs enhanced the survival and accumulation of virus-specific CD8 T cells during vesicular stomatitis virus infection (Swiecki, Gilfillan, Vermi, Wang, & Colonna, 2010). However, the model used to dissect this function has effects beyond the pDC lineage (Swiecki et al., 2014). Conventional dendritic cells directly infected with virus also have the capacity to produce large amounts of type I interferon (Diebold et al., 2003). To our knowledge, no model that impairs pDC development or function has demonstrated that this DC subset is absolutely required to survive viral infections. However, pDCs remain an important therapeutic target for clinical research because they have been implicated in exacerbating a wide range of autoimmune syndromes and hypersensitivity reactions in humans and mice (Ganguly, Haak, Sisirak, & Reizis, 2013; Reizis, Bunin, et al., 2011; Sisirak et al., 2014).

More recent work has suggested that pDCs can regulate initial stages of naïve CD8 T cell priming by cDC1s during viral infections. Using microscopy to locate DC populations and lymphocytes in lymph nodes after vaccinia virus infection, it was observed that pDCs co-localize with CD8 T cells and XCR1$^+$ cDC1s (Brewitz et al., 2017). In *Itgax*-CRE *Ifnar*$^{fl/fl}$ mice, modest reductions in activated CD8 T cells and reduced expression of canonical markers of DC maturation were observed after infection (Brewitz et al., 2017). This result was attributed to the pDC-specific production of type I interferons. Whether pDCs are an essential source cannot be determined unequivocally until a model of pDC-restricted functional or developmental impairment is developed.

7. Models of cDC2 subset-specific identity inform tissue-specific functions

The discovery of a cDC2-restricted progenitor in the bone marrow would suggest there is a mechanism by which cDC2s diverge from a common progenitor of cDC1s and/or pDCs (Grajales-Reyes et al., 2015; Schlitzer et al., 2015). However, no factor has been discovered to be required for specification to the cDC2 lineage. Despite this limitation, transcription factor deficiencies have revealed defects in the development of tissue-resident and migratory cDC2.

All cDC2 subsets express high levels of *Irf4* and low levels of *Irf8* relative to cDC1s (Murphy et al., 2016). *Irf4*$^{-/-}$ mice have severe reductions in CD11b$^+$ and CD4$^+$ cDC2 populations in BM cultures and lymphoid organs, so it was initially concluded that cDC2 development was *Irf4*-dependent (Suzuki et al., 2004; Tamura et al., 2005). However, the inclusion of additional surface markers, such as CD172a (Guilliams et al., 2016), revealed that cDC2s develop in *Irf4*$^{-/-}$ mice albeit with functional impairments (Bajana, Roach, Turner, Paul, & Kovats, 2012; Bajana, Turner, Paul, Ainsua-Enrich, & Kovats, 2016). To date, numerous studies have used *Irf4*$^{-/-}$ mice to implicate cDC2s in the regulation of Th17

polarization, Th2-mediated allergic responses in the lung, and germinal center reactions (Calabro et al., 2016; Krishnaswamy et al., 2017; Persson et al., 2013; Plantinga et al., 2013; Schlitzer et al., 2013). These studies are, however, limited by broad immunological defects in *Irf4*$^{-/-}$ mice beyond the cDC2 subset.

To date, two ontogenetically distinct cDC2 subsets have been defined by their specific ablation in *Itgax*-Cre *Notch2*$^{fl/fl}$ and *Klf4*$^{fl/fl}$ mice. An analysis of the former revealed that a subset of splenic and lymph node cDC2s are required for IL-23-mediated induction of IL-22 by type III innate lymphoid cells (Satpathy et al., 2013). IL-22, in turn, is required for survival of intestinal infection by *Citrobacter* (Vivier et al., 2018). IL-23 is known to be required for Th17 differentiation, and therefore, *Notch2*-dependent DC2s have also been reported to regulate this process (Lewis et al., 2011; Persson et al., 2013; Schlitzer et al., 2013). Analysis of DC populations in *Itgax*-Cre *Klf4*$^{fl/fl}$ mice revealed a requirement for this transcription factor in the development of lymph node and migratory cDC2s (Tussiwand et al., 2015). These mice have deficient type II immune responses, are susceptible to *Schistosoma mansoni* infection, and resistant to house dust mite-induced allergic pathology. These results are also consistent with prior studies that have used *Irf4*$^{-/-}$ mice to model cDC2 function (Plantinga et al., 2013). Therefore, the identification and functional characterization of *Notch2* and *Klf4*-dependent cDC2s support a model in which *Irf4*-expressing cDC2s are composed of at least two developmental and functional subsets.

For over two decades, dendritic cells have been implicated in the regulation of humoral immune responses (Dubois et al., 1997; Krishnaswamy et al., 2017; Ngo, Tang, & Cyster, 1998). cDC2s dependent on lymphotoxin-β receptor signaling for their development are required for IgA production by B-cells in Peyer's patches (Kabashima et al., 2005; Reboldi et al., 2016). Lymphotoxin-β receptor signaling is required for the development of *Notch2*-dependent splenic cDC2s (Satpathy et al., 2013). Multiple groups have reported that circulating red blood cells (RBCs) are surveyed by cDC2s, which are marked by 33D1 expression and located in bridging channels of the spleen (Calabro et al., 2016; Yi et al., 2015). Splenic cDC2s survey autologous RBCs through recognition of CD47 expressed on the cell surface by the receptor Sirpα, which prevents cDC2 activation and phagocytosis. Therefore, when immunized with xenogenic RBC, cDC2s become activated, process, and present RBC antigens (Yi & Cyster, 2013; Yi et al., 2015). Steady state activation of cDC2s by CD47-deficient RBCs, xenogenic RBCs, or CD172-deficiency results in reduced DC2 numbers and impaired antibody production (Hagnerud et al., 2006; Saito et al., 2010; Van et al., 2006).

To further dissect the role of cDC2s in humoral immune responses, various models have been used to impair cDC2 development or function. *Itgax*-CRE *Irf4*$^{fl/fl}$ mice have deficient antibody responses after intravenous immunization with xenogenic RBC, and it was thus concluded that cDC2s regulate humoral immune responses (Calabro et al., 2016). More recently, this effect was attributed to deficient induction of T follicular helper cells and germinal center B cells in the spleens of CD11c-CRE *Notch2*$^{fl/fl}$ mice (Briseno et al., 2018). Dendritic cells can also polarize naïve T cell development through the regulation of cytokine milieus. cDC2-intrinsic expression of the high affinity IL-2 receptor, CD25, was recently demonstrated to regulate local IL-2 availability after RBC immunization (Li, Lu, Yi, &

Cyster, 2016). IL-2 is known to inhibit Tfh polarization through the induction of STAT5, so it was concluded that cDC2-mediated reduction in local IL-2 concentration promotes Tfh differentiation (Ballesteros-Tato et al., 2012; Johnston, Choi, Diamond, Yang, & Crotty, 2012).

DC-intrinsic expression of chemokines regulates T and B cell migration during the initiation of adaptive immune responses (Ngo et al., 1998). cDC1s can regulate lymphocyte trafficking through oxysterol metabolism and the expression of chemokines that signal through receptors, such as EBI2, expressed on germinal center lymphocytes (Hannedouche et al., 2011; Lu, Dang, McDonald, & Cyster, 2017). Chemokine receptor expression is also necessary for DC localization during humoral immune responses. $CCR7^{-/-}$ mice have severe defects in DC and lymphocyte migration in secondary lymphoid organs (Forster et al., 1999), and this model has been used widely to study the immune responses that are dependent on DC migration (MartIn-Fontecha et al., 2003; Ohl et al., 2004).

8. Development will continue to inform function and guide translation

Advances in our understanding of DC development continue to expand the tools with which we can study DC function. This is most notable for the cDC1 lineage, where numerous regulators of development have been identified, and the impacts of their deficiency have been delineated ontogenetically. Early work to uncover the specific function of cDC1s relied on a genetic model in which cDC1s failed to develop. Mortality after immunological challenges identified the immune responses that were dependent on cDC1 function. Until recently, mechanistic research of gene function in cDC1s has thus been limited to in vitro cell lines and cultured primary cells with no known counterparts in vivo. Understanding the development of cDC1s has allowed researchers to develop better methods of generating bona fide cDC1 in vitro. As such, in vitro systems of ontogenetically relevant processes can now be used to develop genetic models that inhibit discrete functions cDC1s in vivo without impacting cDC1 development. Continued research on the development of cDC2s and pDCs will likewise provide better genetic tools. Ultimately, to translate discoveries to the clinic, understanding DC development will provide the essential tools to dissect DC function, and thus novel targets for immunotherapy.

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