Development, Diversity, and Function of Dendritic Cells in Mouse and Human

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The study of murine dendritic cell (DC) development has been integral to the identification of specialized DC subsets that have unique requirements for their form and function. Advances in the field have also provided a framework for the identification of human DC counterparts, which appear to have conserved mechanisms of development and function. Multiple transcription factors are expressed in unique combinations that direct the development of classical DCs (cDCs), which include two major subsets known as cDC1s and cDC2s, and plasmacytoid DCs (pDCs). pDCs are potent producers of type I interferons and thus these cells are implicated in immune responses that depend on this cytokine. Mouse models deficient in the cDC1 lineage have revealed their importance in directing immune responses to intracellular bacteria, viruses, and cancer through the cross-presentation of cell-associated antigen. Models of transcription factor deficiency have been used to identify subsets of cDC2 that are required for T helper (Th)2 and Th17 responses to certain pathogens; however, no single factor is known to be absolutely required for the development of the complete cDC2 lineage. In this review, we will discuss the current state of knowledge of mouse and human DC development and function and highlight areas in the field that remain unresolved.

DEVELOPMENT AND FUNCTION OF MURINE AND HUMAN DENDRITIC CELL SUBSETS

Classical dendritic cells (cDCs) and plasmacytoid DCs (pDCs) make up the two major subsets of DCs that exist in mice and humans. Among cDCs in mice, two major lineages have been identified and are referred to as cDC1s and cDC2s (Guilliams et al. 2014). cDC1s express high levels of IRF8 and are dependent on *Irf8* (Schiavoni et al. 2002; Aliberti et al. 2003), *Batf3*

(Hildner et al. 2008; Edelson et al. 2010), *Id2* (Hacker et al. 2003; Kusunoki et al. 2003), *Nfil3* (Kashiwada et al. 2011), and *Bcl6* for their development (Ohtsuka et al. 2011; Watchmaker et al. 2014). cDC2s express IRF4 and also IRF8 but at levels lower than cDC1 cells, and can be subdivided into at least two functionally distinct subsets that either require the transcription factors Notch2 or KLF4 (Satpathy et al. 2013; Schlitzer et al. 2013; Tussiwand et al. 2015). pDCs also express high levels of IRF8, similar to levels expressed by cDC1s, but depend on the

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transcription factor E2-2 for their development (Cisse et al. 2008). cDCs and pDCs develop from a common progenitor in the bone marrow (BM), known as the macrophage DC progenitor (MDP), which has both DC and macrophage potential (Fogg et al. 2006; Auffray et al. 2009). Restriction to the DC lineage occurs downstream of the MDP at a stage defined as the common DC progenitor (CDP) (Naik et al. 2007; Onai et al. 2013), which can give rise to both pDCs and cDCs. Cells in the gate that defined the CDP were characterized by expression of intermediate levels of c-Kit and by expression of both Flt3 (CD135⁺) and M-CSFR (CD115⁺), differing from the MDP that expresses c-Kit at high levels. The CDP is negative for expression of CD11c and MHC class II molecules. Subsequent studies identified populations within BM that appeared to be a common progenitor of cDCs, termed pre-cDCs, that were first identified in the spleen (Naik et al. 2006) and later independently identified in the BM (Liu et al. 2009). A common marker of both pre-cDCs was the expression of CD11c, and in the BM these cells were defined as expressing Flt3.

Identification of Distinct Committed Progenitors of cDC1 and cDC2 in Murine Bone Marrow

The identification of progenitors with potential for only one type of cDC initially relied on the use of a reporter for the gene *Zbtb46*, which had been previously identified as a marker for cDCs (Meredith et al. 2012; Satpathy et al. 2012a). One study found that the expression of the *Zbtb46*^{gfp} reporter allele by immature cells in the BM was associated with commitment of these cells to the cDC lineage and the exclusion of pDC potential (Satpathy et al. 2012a). However, that study did not examine the various subpopulations of cells expressing *Zbtb46*. Subsequently, it was recognized that *Zbtb46* was expressed heterogeneously in BM cells, by populations of cells that expressed intermediate levels of c-Kit, similar to expression levels in the CDP, but also by cells that lacked c-Kit expression. The majority of the c-Kit^{int} population expressing *Zbtb46*^{gfp} expressed Flt3 (CD135⁺) but did not express M-

CSFR (CD115⁻). However, it was discovered that these two populations represented a divergence in the potential for cDC subsets, with the c-Kit^{int} population being committed to the cDC1 lineage and the c-Kit^{-/lo} population being committed to the cDC2 lineage. These populations were referred to as pre-cDC1 and pre-cDC2 cells, respectively (Grajales-Reyes et al. 2015). An intriguing finding of this study was that the pre-cDC1 cell could develop even in BM of *Batf3*^{-/-} mice, indicating that specification of the pre-cDC1 did not require BATF3. It appeared that the action of BATF3 was rather late in the developmental process and acted to maintain the expression of the *Irf8* gene by interaction with IRF8 at an enhancer site. In a contemporaneous study, single-cell RNA-Seq on pre-DCs, which were defined as Lin⁻CD11c⁺MHCII⁻CD135⁺CD172a⁻, revealed heterogeneity in expression of SiglecH and Ly6C that could be used to identify pre-cDC1 and pre-cDC2 progenitors. Pre-cDC1 cells were identified as SiglecH⁻Ly6C⁻ and pre-cDC2s were SiglecH⁻Ly6C⁺ (Table 1) (Schlitzer et al. 2015). The SiglecH⁺ fraction of the pre-DC was found to give rise to all DC subsets, including pDCs and cDCs, independent of Ly6C expression. The expression of Ly6C indicated the potential for cDC2, but the levels of c-Kit or M-CSFR were unspecified. It is not clear at this time whether the populations described in these two studies represent the same stages of cDC1 and cDC2 specification, and this will await additional analysis. These two studies also differ in the interpretation of whether a clonally identifiable pre-cDC exists. Schlitzer et al. (2015) suggested the existence of a true pre-cDC stage lacking pDC potential but retaining the ability to generate both types of cDCs. Grajales-Reyes et al. (2015) showed that cKit^{int} *Zbtb46*^{gfp} cells that arise from the CDP but lack functional *Batf3* can divert into the cDC2, but not pDC lineage. Thus, conceivably, a natural rate of failure in commitment specification of pre-cDC1 could explain some cDC2 development, but no homogeneous population lacking pDC potential and retaining both cDC1 and cDC2 potential has emerged. Notably, the previously defined pre-cDC in the BM retained substantial pDC potential (Liu et al. 2009) and

Table 1. Markers of lineage committed murine pre-DCs

Surface marker	Pre-cDC1 (Grajales-Reyes et al. 2015)	Pre-cDC1 (Schlitzer et al. 2015)	Pre-cDC2 (Grajales-Reyes et al. 2015)	Pre-cDC2 (Schlitzer et al. 2015)
MHCII	int	—	—	—
Zbtb46	+	?	+	?
CD11c	+	+	+	+
CD24	+	+	—	—
CD115	—	?	+	?
CD117	int	?	—	?
CD135	+	+	+	+
CD172a	int	—	int	—
Ly6C	?	—	?	+
SiglecH	—	—	?	—

Summary of surface marker expression used by two independent groups to identify murine preclassical dendritic cells (cDCs) committed to cDC1 or cDC2 lineages. Question marks indicate that the expression for a marker was not reported for or used to define the population in the study.

was heterogeneous for c-Kit and MHCII expression. Previously defined pre-cDCs and recently defined pre-cDC1 and pre-cDC2 emerge from the BM, can be identified in the blood, and seed peripheral tissues where they are thought to proliferate, although the extent of local proliferation has been difficult to quantify in vivo (Liu and Nussenzweig 2010; Grajales-Reyes et al. 2015). There are a number of remaining unanswered questions regarding the development of these progenitors, particularly with respect to the mechanisms of transcriptional control. Below we will discuss the extension of these findings to human DCs, but first we describe several additional aspects of development and function in murine DCs.

cDC1 Development and Function

Single deficiencies in *Irf8*, *Batf3*, *Nfil3*, *Id2*, and *Bcl6* are all associated with the loss of cDC1s in lymphoid and nonlymphoid tissues (Schiavoni et al. 2002; Aliberti et al. 2003; Hacker et al. 2003; Kusunoki et al. 2003; Hildner et al. 2008; Edelson et al. 2010; Kashiwada et al. 2011; Ohtsuka et al. 2011; Watchmaker et al. 2014). The mechanisms underlying the requirement for these factors are still a matter of active investigation. Recently, BATF3 was found to function in cDC1 development by acting in the maintenance of the high levels of IRF8 that are already expressed

in the CDP. This function of BATF3 is exerted through its interaction with IRF8 at a specific enhancer site in the *Irf8* gene locus that mediates transcriptional autoactivation. This enhancer appears to become activated during specification of the pre-cDC1 cell upon the induction of *Batf3* expression at the final stages of cDC1 specification in the CDP. Deficiency in *Batf3* did not cause a loss of the identifiable pre-cDC1 cells in the BM, indicating that BATF3 is not required for the initial specification process per se but results in the subsequent decay of IRF8 protein levels and diversion of the specified pre-cDC1 cells into the cDC2 lineage (Grajales-Reyes et al. 2015). This result explains the loss of cDC1s in all lymphoid and nonlymphoid tissues in *Batf3*^{−/−} mice under homeostatic conditions. This also explains why cDC1s in *Batf3*^{−/−} mice can be restored under other conditions, such as during infection or treatment with interleukin (IL)-12 through the induction of *Batf* that can compensate for *Batf3* deficiency and rescue cDC1 development (Tussiwand et al. 2012). In *Itgax-Cre Irf8*^{fl/fl} mice, in which *Irf8* is deleted downstream of the CDP, cDC1s exhibit reduced survival in peripheral tissues, demonstrating that *Irf8* is also required during the terminal stages of cDC1 development (Sichien et al. 2016).

With the knowledge that cDC1 development is *Irf8*-dependent, observations that either

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deficiency in *Irf8* or DC-dependent IL-12 production results in susceptibility to *Toxoplasma gondii* shed light on the specialized functions of cDC1 cells (Scharton-Kersten et al. 1997; Liu et al. 2006). It was first demonstrated that these defects were intrinsic to cDC1s through the generation of *Batf3*^{-/-}:*Il12p40*^{-/-} mixed BM chimeras, in which IL-12 production is only deficient in cDC1s (Mashayekhi et al. 2011). It was also revealed by using *Batf3*^{-/-} mice that cDC1s are required for cross-presentation of exogenous antigen to CD8 T cells, which in turn is required for antiviral and antitumor responses (Hildner et al. 2008). While normally conferring protection to pathogens, cDC1s can also confer susceptibility in certain cases, such as in the setting of blood-borne infection by *Listeria monocytogenes*. In this particular setting, cDC1s act as the primary cellular target of infection that leads to the spread of the pathogen into the lymphoid areas of the spleen, where massive cellular loss occurs as a result (Edelson et al. 2011a). Surprisingly, mice lacking BATF3, and thus lacking cDC1s, were remarkably resistant to intravenous infection by *L. monocytogenes* (Edelson et al. 2011a). Although the adaptive function of cDC1s is largely considered to be restricted to immune responses mediated by CD8 T cells and T helper (Th)1 cells, *Batf3*^{-/-} mice have enhanced Th2 responses to helminth infection, a phenomenon attributed to loss of constitutive expression of IL-12 by DC1s (Everts et al. 2016).

cDC2 Development and Function

Although several transcription factors are implicated in regulating cDC2 development, there is, to our knowledge, no mutant mouse model in which cDC2 development is selectively ablated. This is in contrast to several single transcription factors whose deletion can ablate cDC1 development. RelB was the first transcription factor to be implicated in the development of cDC2s (Burkly et al. 1995; Weih et al. 1995). Germline deletion of *Relb* in mice causes a multifaceted phenotype that includes splenomegaly, extramedullary hematopoiesis, multiorgan inflammation, myeloid hyperplasia, and disturbed de-

velopment of thymic and splenic cDCs (Burkly et al. 1995; Weih et al. 1995). Initial studies performed to determine the cell-intrinsic requirements for RelB in DC development claimed that cDCs did not develop in wild-type (WT) chimeras reconstituted with *Relb*^{-/-} BM; however, little to no evidence was ever provided in these studies to support such a statement (Burkly et al. 1995; DeKoning et al. 1997; Gerloni et al. 1998a,b). Subsequent independent analysis showed that thymic CD8α⁺ cDC1s develop normally in *Relb*^{-/-} WT chimeras, but suggested that there was a cell-intrinsic requirement for RelB in the development of CD8α⁻Dec205⁻ cDC2s (Wu et al. 1998). Another report confirmed reduced cDC numbers in *Relb*^{-/-} mice but did not establish a cell-intrinsic requirement for their development or function (Kobayashi et al. 2003). Very recently, our analysis concluded that the majority of cDCs show no cell-intrinsic requirement for RelB for their development (Briseno et al. 2017) with one exception. There was a cell-intrinsic requirement for RelB only in the development of the CD4⁺Esam⁺ cDC2 subset of the spleen (Briseno et al. 2017), a subset that is also dependent on Notch2 signaling (Satpathy et al. 2013) and lymphotoxin β (LT-β) receptor signaling (Kabashima et al. 2005). This subset of cDC2 cells appears to represent a terminal maturational stage of cDC2 cells (Satpathy et al. 2013) that develops in response to Notch ligands expressed in specific lymphoid tissue niches (Fasnacht et al. 2014). This similarity between the phenotypes caused by deficiency in *Relb* and LT-β receptor might suggest that RelB could act downstream of this receptor in the final maturation of cDC2 progenitors in such lymphoid niches. However, the majority of cDCs in lymph nodes and peripheral organs showed no cell-autonomous requirement for RelB in their development. There was, however, a role for RelB in non-hematopoietic tissues that regulated the myeloid compartment. Specifically, *Relb*^{-/-} recipient chimeras reconstituted with WT BM showed a similarly abnormal myelopoiesis to that observed in germline *Relb*^{-/-} mice, indicating that the initially reported abnormality of myeloid cells in *Relb*^{-/-} mice was a result of loss of

RelB in nonhematopoietic, radio-resistant cells. Although *Relb*^{-/-} cDCs are able to activate T cells against cell-associated antigens (Briseno et al. 2017), a role for RelB in other cDC functions has not been excluded, although these are unknown at present. A novel floxed allele for conditional deletion of *Relb* should facilitate such studies (De Silva et al. 2016).

The functional specialization of cDC2 subsets has been revealed using models of transcription factor deletion. *Itgax*-Cre-mediated deletion of *Notch2* and the signaling partner, *Rbpj*, results in the loss of cDC2s in the spleen that are CD11b⁺ESAM⁺, and cDC2s in the intestinal lamina propria and mesenteric lymph nodes that are CD103⁺CD11b⁺ (Satpathy et al. 2013). Loss of this subset is associated with susceptibility to *Citrobacter* infection. Mortality at the early time point of day 10 after infection in *Notch2*^{fl/fl} *Itgax*-Cre mice suggested a role for the *Notch2*-dependent cDC2 subset in innate defense, in addition to its expected role in adaptive immunity. Using mixed chimeras of *Itgax*-Cre *Notch2*^{fl/fl} and *Il23a*^{-/-} BM, it was found that IL-23 production by the *Notch2*-dependent cDC2 subset is required during *Citrobacter* infection (Satpathy et al. 2013). IL-23 is known to activate ILC3 cells for production of IL-22, a cytokine that is required to maintain the barrier function of intestinal epithelial cells (Zheng et al. 2008; Sonnenberg and Artis 2015). Reduced numbers of Th17 cells has also been observed in models where the development or function of cDC2s is impaired. *Itgax*-Cre *Irf4*^{fl/fl} mice show a defect in the production of Th17-polarizing cytokines on immunization and reduced Th17 populations at homeostasis (Persson et al. 2013; Schlitzer et al. 2013). A specific deficiency in transforming growth factor β (TGF- β) or IL-6 in CD11c-expressing cells is also sufficient to reduce Th17 polarization following infection with *Streptococcus pyogenes* (Persson et al. 2013; Schlitzer et al. 2013; Linehan et al. 2015). A specific requirement for cytokine production by cDC2s, rather than other CD11c-expressing subsets, in Th17 polarization has not been established.

At steady state, expression of *Klf4* is required for the development or function of a subset of

migratory cDC2 cells that are CD11b⁻CD24⁻ in the skin-draining lymph node and CD24⁺CD172a⁺Mgl2⁺ in the lung. A loss of these cells in *Itgax*-Cre *Klf4*^{fl/fl} mice correlates with enhanced susceptibility to helminth infection and enhanced lung inflammation during house dust mite challenge (Tussiwand et al. 2015). These results are consistent with previous studies that used an *Mgl2*-DTR and *Itgax*-Cre-mediated deletion of *Irf4* that attributed reduced Th2 responses to *Irf4*-expressing cDC2s (Gao et al. 2013; Kumamoto et al. 2013). Still, it is not clear that the missing population is directly responsible for inducing Th2 responses, and the mechanism underlying these phenomena are unknown. Recent studies suggest that ILC2s promote the migration of cDCs to draining lymph nodes, and that cDC2s express chemokines that attract memory Th2 cells on rechallenge (Halim et al. 2014, 2016). Further, normal Th2 responses are driven by cytokines, including IL-13, that are produced by ILC2 cells in response to IL-25 produced in tissues, for example, by epithelial tuft cells in response to certain stimuli (Van Dyken et al. 2016; von Moltke et al. 2016). Thus, it is conceivable that Th2 responses may rely on T cells that reach the tissues in a sufficiently nonpolarized state to respond to this “tissue checkpoint” (Van Dyken et al. 2016). Because BATF3- and *Notch2*-dependent DCs have been associated with Th1 and Th17 responses, perhaps KLF4-dependent DCs simply provide for activation of T cells without strong polarization, allowing for flexible T-cell responses in tissues.

pDC Development and Function

E2-2, encoded by *Tcf4*, is a member of the E family of basic helix-loop-helix transcription factors (Kee 2009). In both mice and humans, E2-2 is required for the specification of CDPs to pDCs (Cisse et al. 2008). Induced deletion of E2-2 in mature pDCs results in the acquisition of cDC-like properties, such as dendritic morphology, MHCII and CD8 α expression, and the ability to induce proliferation of allogeneic CD4⁺ T cells (Ghosh et al. 2010). Deletion of E2-2 in pDCs also induces the expression of ID2.

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MTG16, a transcriptional cofactor of the ETO protein family, represses the expression of ID2 in pre-DCs and mature pDCs (Ghosh et al. 2014). The proteins encoded by *Tcf4* are expressed as multiple isoforms (Corneliusson et al. 1991), TCF4s (short) and TCF4L (long) (Sepp et al. 2011). TCF4L contains activation domain 1 (AD1), which can interact with both p300 and the corepressor RUNX1T1 (Zhang et al. 2004). Within the immune system, TCF4s is expressed in many cells, including cDCs, B cells, and pDCs; however, TCF4L expression is restricted to pDCs (Grajowska et al. 2017). Loss of TCF4L caused a reduction of pDCs in the BM and spleen similar to that observed in *Mtg16*^{-/-} mice. The induction of *Tcf4* expression in pDCs is regulated by a proximal pDC-specific 3' enhancer that requires TCF4 to maintain a positive feedback loop. TCF4L induction occurs at the CDP stage of development, but the stage at which it is required for development remains unclear. This would be aided by the identification of the clonogenic pDC progenitor; however, so far, there has only been identification of populations of BM cells that show relative enrichment for pDCs, and no population that is clonogenically restricted to the pDC lineage has been reported to date (Schlitzer et al. 2011). Similarly, the transcriptional basis for pDC specification and commitment awaits identification.

One mechanism proposed for pDC specification is the expression of ID2, which is required for cDC1 development. Recently, we and others identified *Zeb2*, a Zinc-finger homeodomain transcription factor (Vandewalle et al. 2009) to be required for pDC development (Scott et al. 2016; Wu et al. 2016b). Germline deletion of *Zeb2* causes embryonic lethality in mice as a result of its action during the epithelial-mesenchymal transition (Higashi et al. 2002; Van de Putte et al. 2003), which involves the repression of E-cadherin (Comijn et al. 2001; Vandewalle et al. 2005). In the nervous system, ZEB2 regulates myelination by modulating the actions of Smad proteins, which are activated members of the TGF- β superfamily known as bone morphogenic proteins (Weng et al. 2012). In oligodendrocyte precursors, *Zeb2* expression is low and activated Smads bind P300, a coactivator histone

acetyltransferase, inducing expression of negative regulatory genes such as *Id2* and *Hes1*. However, in differentiating oligodendrocytes, OLIG1 and OLIG2 induce the expression of ZEB2, which binds to and represses Smad-P300 complexes thus blocking *Id2* and *Hes1* expression (Weng et al. 2012). Within DC development, ZEB2 appears to act as a negative regulator of ID2. We found that deletion of *Zeb2* in DCs using *Itgax*-Cre caused slightly higher expression of *Id2* in cDC2s compared with *Id2* expression in WT cDC2 cells. Overexpression of *Zeb2* in BM cultures stimulated with FLT3L caused strongly increased pDC development while restricting the frequency of cDC1 cells (Wu et al. 2016b). The role of ZEB2 in cDC2s is still unclear. If specification to the pDC and cDC lineages is dependent *Zeb2* and *Id2*, respectively, it is unclear how cDC2s develop in *Id2*^{-/-} mice (Hacker et al. 2003; Kusunoki et al. 2003). In summary, it is unclear currently whether ID2 acts simply to exclude pDC potential from cells arising from the CDP population, for example, by preventing runaway E2-2 expression (Grajowska et al. 2017) or, alternatively, whether it acts to support cDC1 development in some way. In either case, the actual mechanism has not been identified.

Other factors have been implicated in pDC development. Deletion of *Runx2*, a Runt family transcription factor that is required for osteoblast development (Komori et al. 1997; Otto et al. 1997), causes reduced expression of CCR5 on pDCs, thus impairing their egress from the BM to the periphery (Sawai et al. 2013). Previously, it was thought that deletion of *Irf8* prevented pDC development (Schiavoni et al. 2002). However, a recent study showed that pDCs develop in *Itgax*-Cre *Irf8*^{fl/fl} mice but exhibit an abnormal phenotype and altered transcriptional profile (Sichien et al. 2016). This result does not rule out a requirement for *Irf8* in the development of pDCs prior to the expression of CD11c; however, given the altered phenotype of pDCs in *Itgax*-Cre *Irf8*^{fl/fl} mice, it is conceivable that pDCs may still be present in *Irf8*^{-/-} mice. A reevaluation of the dependence on *Irf8* for pDC development may thus be warranted.

Human DCs

Recent efforts to identify human counterparts of murine DCs suggest that their development is conserved across species (Dutertre et al. 2014). The current understanding of the cellular stages of DC development in mice, particularly progenitors developing in the BM, has been reviewed recently (Murphy et al. 2016). Identification of the human counterparts has been challenging because of relative limitations in access to samples such as BM compared with mice. Human cDC1s are identified by the expression of CD141, Clec9a, and XCR1 (Bachem et al. 2010; Crozat et al. 2010; Poulin et al. 2010). Like murine cDC1s, these cells express IRF8, produce IL-12, and have superior capacity to cross-present (Haniffa et al. 2012). Human cDC2s can be identified by the expression of CD1c and BDCA1, and, like their mouse counterparts, express IRF4, produce IL-23, and induce differentiation of Th17 cells in response to *Aspergillus fumigatus* (Schlitzer et al. 2013). Recently, cDC2 cells in peripheral blood were segregated into two distinct groups based on CD5 expression (Yin et al. 2017). CD5^{high} cDC2s expressed high levels of IRF4 and were potent inducers of T-cell activation. The ontogeny of CD5^{lo} cells, however, appears unclear. CD5^{lo} DCs express high levels of *MafB*, which in mice is highly expressed in monocytes and macrophages but not cDCs (Satpathy et al. 2012b; Wu et al. 2016a). A large cohort of human lymphoid tissue samples was used to confirm the broad tissue distribution of cDC1s and cDC2s and the conservation of cDC migratory phenotypes between mice and humans based on the expression of CCR7 and higher MHCII (Granot et al. 2017).

With the application of single-cell RNA-Seq (scRNA-Seq), several studies have identified human DC progenitors in BM and blood. Three independent studies described human pre-cDC progenitors with cDC1 and cDC2 potential (Breton et al. 2015a,b; See et al. 2017; Villani et al. 2017), using different surface markers (Table 2). However, whether these populations are related has not been tested. A side-by-side comparison of pre-cDCs identified by See et al.

Table 2. Markers of humans pre-cDCs in peripheral blood

Surface marker	Pre-cDC (Breton et al. 2015a,b)	Pre-cDC (Villani et al. 2017)	Pre-cDC (See et al. 2017)
HLA-DR	+	+	+
CD11c	lo		lo
CD14	—	—	—
CD34	—	int	—
CD45RA	+	+	+
CD100	?	+	?
CD115	—	—	?
CD116	+		+
CD117	+	+	—
CD123	int	—	+
CD135	+	—	+

Summary of surface marker expression used by three independent groups to identify distinct populations of pre-classical dendritic cells (cDCs) in peripheral blood from humans. Question marks indicate that the marker's expression was not reported in the study.

and Breton et al. showed that the former was more abundant, had higher expression of CD303, and lower expression of CD117 (See et al. 2017). However, it has been suggested that pre-cDCs are heterogeneous and composed of cells already specified to each subset of the cDC lineage, similar to that observed in mice (Breton et al. 2016; Grajales-Reyes et al. 2015). Along these lines, progenitors with predominantly cDC1 or cDC2 potential have also been identified (Table 3). The first set of committed pre-cDC progenitors were distinguished based on surface expression of CD172a, cDC1 progenitors being CD172a⁺ and cDC2 progenitors CD172a⁺ (Breton et al. 2016). See et al. (2017) independently defined two populations of pre-cDCs that are CD33⁺CD45RA⁺CD123^{lo}. The first is CADM1⁺ and gives rise to cDC1s, and the second is CD1c⁺ and gives rise to cDC2s (See et al. 2017). As defined by See et al. (2017), circulating pre-cDC1 and pre-cDC2 cells were morphologically similar to mature cDC1 and cDC2, secreted cytokines after TLR activation, and induced T-cell proliferation during allogeneic responses in vitro.

A novel DC population in circulation identified by scRNA-Seq has recently been proposed and is referred to as the AS-DC based on the

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Table 3. Markers of cDC1 and cDC2 committed human pre-cDCs

Surface marker	Pre-cDC1 (Breton et al. 2016)	Pre-cDC1 (See et al. 2017)	Pre-cDC2 (Breton et al. 2016)	Pre-cDC2 (See et al. 2017)
HLA-DR	+	+	+	+
Cadm1	?	+/int	?	—
CD1c	—	—	—	+
CD14	—	—	—	—
CD33	?	+	?	+
CD34	—	—	—	—
CD45RA	+	+	+	+
CD115	—	?	?	?
CD116	+	?	+	?
CD117	+/int	?	+/int	?
CD123	—	+/-	—	—
CD135	+	+	+	+
CD172a	—	?	+	?

Summary of flow cytometry analysis of surface marker expression used to identify classical dendritic cell (cDC)1 and cDC2 committed pre-cDCs in humans. Question marks indicate the expression for a marker that was not reported for or used to define the population in the study.

expression of AXL and SIGLEC6 (Villani et al. 2017). The gene signatures observed for this population clustered between pDCs and cDC2s. The authors of the study suggest this population does not represent an intermediate stage of pre-cDCs because DC progenitors do not induce the expression of AXL or SIGLEC6 in culture (Villani et al. 2017). However, the pre-cDC reported by See et al. (2017) expresses the genes that encode these markers. Interrogation of the molecular mechanisms that control human cDC development is limited but gene expression analysis of the progenitors identified to date suggest conservation between mouse and human. For example, specification of cDC1 and cDC2 progenitors is associated with the differential expression of known regulators of mouse DC development, including BATF3, ID2, TCF4, IRF4, ZEB2, and IRF8 (Breton et al. 2016; See et al. 2017).

Human pDCs produce high levels of type I interferons (IFNs) during responses to viral infection (Cella et al. 1999; Siegal et al. 1999). They can also activate CD4⁺ and CD8⁺ T cells in response to influenza virus (Fonteneau et al. 2003). However, heterogeneity within the bulk pDC population was later recognized using CD2 as a marker to distinguish two distinct pDC populations (Matsui et al. 2009). CD2⁺ pDCs

secrete high levels of IL-12p40, induce surface expression of CD80, and induce proliferation of naïve allogeneic CD4⁺ T cells. These cells more closely resemble cDCs than pDCs. This CD2⁺ population was further refined using CD5 and CD81 to identify the pDC population capable of secreting IL-12 and activating CD4⁺ T cells (Zhang et al. 2017a). A separate study showed that CD56 expression identified a myeloid DC population within the CD2⁺ pDC gate. This novel population did not produce IFN- α , and instead secreted IL-12 and activated T cells. Transcriptomic analysis showed that CD2⁺ CD56⁺ pDCs were more closely related to cDCs than to pDCs (Yu et al. 2015). Further, transcriptomic analysis of CD56⁺ pDCs showed they were closely related to blastic plasmacytoid DC neoplasms (BPDCNs). These observations were further confirmed by Villani et al. (2017), in which the AS-DC population shared some transcriptomic characteristics with pDCs. Functionally, they were potent inducers of T-cell proliferation and secreted high levels of IL-12. These multiple lines of evidence suggest that the cDC-like function attributed to a subset of human pDCs is the result of analysis of heterogeneous populations composed of cDCs and pDCs in early studies of human pDC function.

CONTEMPORARY ANALYSIS OF PARADIGMS IN cDC DEVELOPMENT AND FUNCTION

Identification of cDCs in Vivo

Shared surface marker expression among cells of the myeloid lineage has complicated the discrimination of DC subsets from other myeloid lineages. Recent analyses have proposed a simplified set of markers to discriminate DC subsets across tissues by defining cDCs as Lin[−]CD11c⁺MHCII⁺CD26⁺CD64[−], among which cDC1s and cDC2s can be identified as XCR1⁺ and CD172a⁺, respectively (Guilliams et al. 2016). Surface-marker-independent methods of discriminating lineages have been helpful in resolving the origin of myeloid cells in vivo. Expression of the transcription factor, ZBTB46, is restricted to the cDC lineage and can be used to identify cDCs and their progenitors in lymphoid and nonlymphoid tissues (Satpathy et al. 2012a; Grajales-Reyes et al. 2015). Alternatively, the transcription factor MafB is expressed by cells of the monocyte and macrophage lineage (Aziz et al. 2009; Gautier et al. 2012). A novel lineage-tracing reagent, MafB-mCherry-Cre mice, marks cells that express MafB (mCherry⁺) or have expressed MafB during development (YFP⁺ when crossed to R26-stop-YFP mice), and can thus be used in combination with Zbtb46 to discriminate between macrophage and DC lineages in vivo (Wu et al. 2016a). Interestingly, it was found that, among the tissues examined, Langerhans cells (LCs) in skin-draining lymph nodes were the only lineage to express *Zbtb46* and also be marked by *Mafb*-driven lineage tracing.

Mo-DCs and GM-DCs

Numerous studies have suggested that under inflammatory conditions monocytes have the potential to differentiate into cDCs. From in vitro studies, it is known that monocytes from mice or humans cultured with granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4, referred to as Mo-DCs, acquire characteristics of cDCs (Caux et al. 1992; Inaba et al. 1992, 1993; Romani et al. 1994; Sallusto

and Lanzavecchia 1994). Similarities include the expression of canonical surface markers, such as CD11c and MHCII (Leon et al. 2004), and DC-specific transcription factors, such as *Zbtb46* and *Mycl1* (Satpathy et al. 2012a; Wumesh et al. 2014). Upon treatment of GM-CSF, monocytes rapidly induce the expression of IRF4 (Lehtonen et al. 2005), which is required for their differentiation into cDC-like cells that express *Zbtb46* and MHCII (Briseno et al. 2016). Mo-DCs have the ability to cross-prime CD8 T cells to cell-associated antigens in vitro; however, cross-presentation by the subset specialized for this activity in vivo, cDC1s, is *Irf4*-independent (Vander et al. 2014; Briseno et al. 2016). GM-CSF-derived DCs (GM-DCs) have been used extensively in studies surveying DC function. Many of the known actors involved in cross-presentation were first identified in GM-DCs and are reviewed here (Theisen and Murphy 2017). However, it was recently reported that BM cultures stimulated with GM-CSF produce heterogeneous populations and has thus casted doubt over physiological relevance GM-DCs to in vivo cDC subsets (Helft et al. 2015). To obtain populations of pDCs and cDCs that more closely resemble in vivo counterparts, an alternative in vitro culture system that uses whole BM or purified progenitors in Flt3L was developed (Naik et al. 2005). We recently identified *Rab43* to be involved in the cross-presentation of cell-associated antigen by cDC1s but not by GM-DCs (Kretzer et al. 2016). Therefore, it may be necessary to evaluate the function of molecules previously reported in GM-DCs to regulate vesicular trafficking and cross-presentation, including but not limited to RAC2 (Savina et al. 2009), RAB11A (Nair-Gupta et al. 2014), RAB3B (Zou et al. 2009), and SEC22B (Cebrian 2011).

The precise role of GM-DCs in promoting CD8⁺ T-cell responses via cross-presentation is unclear because *Zbtb46*-expressing Mo-DCs have yet to be distinguished in vivo from bona fide CD11b⁺ DCs, and thus a model to selectively deplete them is unavailable. Recent work that replicated in vivo models of putative Mo-DC differentiation by house dust mite challenge did not identify *Zbtb46*-expressing cells that

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were marked by *MafB*-driven lineage tracing (Wu et al. 2016a). Therefore, the developmental origins of the Mo-DCs in vivo remain elusive. Notwithstanding, GM-DCs have been demonstrated to be a viable option in the generation of tumor vaccines (Linette and Carreno 2013). Human Mo-DCs generated with GM-CSF and IL-4 have been used as the basis for therapeutic cancer vaccines (Palucka and Banchereau 2013; Carreno et al. 2015). Vaccines based on human Mo-DCs pulsed with tumor-specific peptides can initiate CD8 T-cell responses and induce clinical responses in melanoma, renal cell carcinoma, and malignant glioma (Nestle et al. 1998; Holtl et al. 1999; Thurner et al. 1999; Timmerman et al. 2002). Human Mo-DCs generated ex vivo can also elicit broad CD8⁺ T-cell responses against tumor antigens, and to a class of subdominant neoantigens in patients with melanoma (Carreno et al. 2015).

cDC Maturation

An additional area of study to emerge from the use of GM-DCs is DC maturation. The term “mature” was first used to describe the adherent fraction of DCs isolated from the spleen of mice (Steinman and Cohn 1973). The process of “maturation” was later described as the acquisition of T-cell stimulatory capacity of LCs and DCs after isolation and ex vivo culture (Schuler et al. 1985; Witmer-Pack et al. 1987; Heufler et al. 1988). The capacity to stimulate T cells was correlated with the induction of costimulatory molecules, such as CD80 and CD86 (Inaba et al. 1994), and chemokine receptors, such as CCR7 (Sallusto et al. 1998; Sozzani et al. 1998). Immature DCs in vitro most closely resemble resident DCs in vivo and are identified as CD11c^{hi} MHCII⁺. Mature DCs in vitro most closely resemble migratory DCs in vivo and are identified as CD11c⁺ MHCII^{hi}. Consistent with this correlation, migratory DCs in vivo express elevated levels of the canonical maturation markers CCR7, CD80, CD86, and CD40. CCR7 is required for the migration of cDCs to draining lymphoid organs (Forster et al. 1999; Ohl et al. 2004), CD80 and CD86 are required for stimulation of naïve T cells (Steinman et al. 2003), and

CD40 is required to receive CD4 T-cell help (Bennett et al. 1998; Schoenberger et al. 1998). Therefore, the study of maturation in vitro has led to important in vivo discoveries regarding fundamental DC biology. However, recent analysis of maturation in vivo calls for a revision of previously established paradigms regarding functional differences between immature and mature DCs.

Low expression of costimulatory molecules on immature DCs formed the basis for a hypothesis that immature DCs are specialized at tolerance induction and have thus been referred to as tolerogenic (Morelli and Thomson 2007). Recent in vivo evidence is contrary to this distinction. It was recently shown that mature cDC1s are the sole population capable of cross-presenting thymic epithelial-cell-derived self-antigens, and that BATF3-dependent cDC1s are required to induce a subset of *Aire*-dependent natural regulatory T (Treg) cells (Perry et al. 2014; Ardouin et al. 2016). Peripheral Treg induction in the small intestine lamina propria is also induced by cDC1s that have taken up host-derived antigen and migrate to draining lymph nodes. These cells express higher levels of *Ccr7* and undergo transcriptional reprogramming associated with maturation (Cummings et al. 2016). This is consistent with results from the examination of draining lymph nodes of the oral mucosa, where migratory cDC1s are most efficient at inducing oral tolerance to dietary antigens (Esterhazy et al. 2016). Contrary to a role for cDC1s in the induction of tolerance, cDC1s can also be essential for the initiation of autoimmunity in mice with genetic backgrounds predisposed to the development of diabetes (Ferris et al. 2014).

Given that functional distinctions between immature and mature DCs may not be consistent with phenomena that occur in vivo, the process of maturation may be better conceptualized as a stage of cDC development. cDC progenitors are known to egress from the BM specified to either the cDC1 or cDC2 lineage (Grajales-Reyes et al. 2015). In peripheral tissues, immature cDCs exhibit phenotypes associated with cell division and proliferation (Liu et al. 2007). Work to identify the factors that

regulate DC proliferation remains an active area of research. In secondary lymphoid organs, deficiency in the DC-specific transcription factor, L-Myc, results in a reduction in the number of cDC1s, reduction in DNA replication associated with cell division, reduction in priming of antigen-specific CD8 T cells, and enhanced resistance to infection with *L. monocytogenes* (Wumesh et al. 2014). The growth factor, Flt3L, has been shown to expand BM progenitors of cDCs and increase the population size of cDCs in lymphoid organs (Waskow et al. 2008). Although a requirement for GM-CSF in cDC1 development is debated (Edelson et al. 2011b; Greter et al. 2012), treatment with GM-CSF in vivo and in vitro in combination with Flt3L is sufficient to expand populations of cDCs (Daro et al. 2000; Mayer et al. 2014).

The extent to which local growth factor concentrations and milieus influence proliferation of cDCs at steady state and during inflammation in peripheral tissues is not well understood, and may be made redundant by constant recruitment of progenitors from the BM (Liu and Nussenzweig 2010). Whole transcriptome analysis of mature CCR7⁺ and immature CCR7⁻ cDC1s sorted from the thymus and spleen at steady state revealed broad transcriptional reprogramming that includes differential expression of genes associated with exit from the cell cycle (Ardouin et al. 2016). Similar transcriptional changes have been detected in cDCs from a variety of tissues when resident and migratory counterparts are compared (Manh et al. 2013). Identification of cells that have undergone cell-cycle exit or entered a state of quiescence is commonly used to uncover stages at which terminal differentiation occurs during the ontogeny of cellular lineages (Massague 2004; Buttitta and Edgar 2007; Collier 2011). Therefore, cell-cycle exit on cDC maturation suggests that this process represents a terminal stage in the developmental program of cDCs. The factors necessary to induce maturation in vivo remain largely unknown, and much of the work conducted to date has focused on the cell-extrinsic influence of host- and commensal-derived stimuli.

Both host- and microbiota-derived factors have been reported to be sufficient to induce

DC maturation. In a model of vaccination using mice deficient in IFNAR, it was shown that type I IFN in response to poly-IC acted directly on DCs to induce maturation of splenic DCs and induce Th1 immunity to a model antigen of HIV (Longhi et al. 2009). In models of viral infection and tumor rejection, the action of type I IFN on cDCs was required for optimal CD8 T-cell priming and Th1-cell polarization (Brewitz et al. 2017; Diamond et al. 2011; Fierres et al. 2011). Recent whole transcriptome analysis has demonstrated that transcriptional reprogramming is conserved between maturation at homeostasis or under inflammatory conditions of poly-IC injection or viral infection, and occurs independently of IFNAR signaling (Ardouin et al. 2016). Although signaling through IFNAR may be sufficient to induce maturation in the spleen (Longhi et al. 2009), where at least 90% of DCs exhibit an immature phenotype (Ardouin et al. 2016), such signals are not necessary to execute the transcriptional program that occurs during this process.

Signaling cascades initiated by engagement of the receptors for IL-1 β , tumor necrosis factor α (TNF- α), CD40L, and LT converge on activation of canonical and noncanonical nuclear factor (NF)- κ B (Jost and Ruland 2007). As discussed above, it was recently shown that a requirement of RelB in the development of cDCs is largely cell-extrinsic, with the exception of a splenic cDC2 subset that is also Notch2- and LT β R-dependent (Kabashima et al. 2005; Satpathy et al. 2013; Briseno et al. 2017). However, this does not rule out a DC-intrinsic role for RelB or the remaining NF- κ B family members in cDC maturation. In vivo analysis of p50-deficient mice showed no effect on the expression of CD80 or CD86; however, they have a defect in Th2 cell differentiation during helminth infection, which is now known to be regulated by the KLF4-dependent cDC2 subset (Artis et al. 2005; Tussiwand et al. 2015). An independent study also showed that cRel, p50, and RelA are dispensable for development of cDCs and the expression of CD80 and CD86. However, the deletion of p50 and RelA together led to a significant loss of CD11c⁺ cells in the spleen (Ouaaz et al. 2002). Therefore, NF- κ B family members may have

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compensatory roles in cDC development and function. Defining such combinatorial complexity in NF- κ B activity has been difficult to define in vivo, because up to 15 dimer combinations are possible with 13 reported to date (Smale 2012; Zhang et al. 2017b). Modules of genes that are known NF- κ B targets are differentially expressed during maturation (Manh et al. 2013; Ardouin et al. 2016); however, definition of the cell-intrinsic requirements for NF- κ B in cDC function remains a hurdle to overcome in this field.

Homeostatic interactions between commensal microbiota and the host-immune system have been linked to various immunological disorders in patients with mutations of pattern-recognition receptors (PRRs) (Hooper et al. 2012). Direct signals from microbiota acting on DCs at steady state and during infection have been suggested to regulate DC maturation (Steinman et al. 2003). Although there is mounting evidence for the regulation of immune homeostasis through interactions between the host and commensal microbiota (Belkaid and Hand 2014), evidence for the regulation of DC maturation is limited. DC-specific deletion of *Traf6*, which encodes a signaling adaptor downstream of various PRRs, results in defective inflammatory cytokine production on stimulation with CpG and LPS. These mice also develop spontaneous inflammation of the small intestine that is associated with aberrant Th2 cell priming, which can be rescued by treatment with antibiotics (Han et al. 2013). *Traf6*^{-/-} mice have defective induction of maturation markers on DCs in vivo when treated with LPS or CD40L and, therefore, it was proposed to be necessary for DC maturation (Kobayashi et al. 2003). However, in single and double knockout mice of *MyD88* and *Ticam1*, no effect on the development or maturation of DCs was observed (Wilson et al. 2008). Such conflicting results are difficult to interpret in light of complex cross talk that may establish redundancy in signaling pathways downstream of PRRs (Lee and Kim 2007). Therefore, ablation of commensal microbiota is widely used as a strategy to probe the impact of steady-state microbial signals on immune homeostasis. To that end, WT specific pathogen-free (SPF) and

germ-free mice showed no significant differences in the core transcriptional program associated with maturation in vivo for thymic cDC1s (Ardouin et al. 2016). In addition, the core maturation programs that occur at homeostasis or under inflammation induced by poly-IC or STAg overlap broadly (Ardouin et al. 2016). As opposed to a model that focuses on cell-extrinsic stimuli, representation of maturation as a cell-intrinsic developmental program can provide an alternative framework to discover novel mechanisms that regulate DC development and function.

CONCLUSION

In summary, analysis of the molecular events that underlie distinct forms of DCs in the mouse have advanced over the past 8 years, with the identification of several transcription factors required for some, but not all, DC subsets. Notably, while several factors appear to be required for cDC1 and pDC development, there has been no single factor whose ablation selectively prevents cDC2 development. It is true that Notch2 is required for the normal functioning of cDC2 in response to certain pathogens and that KLF4 is required for cDC2 support of Th2 type responses, but, in each case, cDC2 cells develop in the absence of these factors. It is not clear that these results mean that cDC2 development is a “default” pathway, because it may turn out that a mechanism will be found that is necessary for development of all forms of cDC2. Progress arising from analysis of the murine system has also provided a basis for analysis of human DCs, which now are recognized as being structurally similar to their murine counterparts, at least in certain fundamental ways. These studies in both systems promise to provide a basis for future rational therapeutic interventions to complement the current progress in immunotherapy.

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