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Nat Immunol. Author manuscript; available in PMC 2013 December 01.

Published in final edited form as:
Nat Immunol. 2012 December ; 13(12): 1145–1154. doi:10.1038/ni.2467.

Re(de)fining the dendritic cell lineage

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Abstract

Dendritic cells (DCs) are essential mediators of the innate and adaptive immune response. Studying these critical cells has been complicated by their similarity to other hematopoietic lineages, particularly monocytes and macrophages. Recent progress has been made in three critical areas of DC biology: the characterization of lineage-restricted progenitors in the bone marrow, the identification of cytokines and transcription factors required during differentiation, and the development of genetic tools to visualize and deplete DCs *in vivo*. Collectively, these studies have clarified the nature of the DC lineage and provided novel insights into their function during health and disease.

Keywords

dendritic cell; macrophage; transcription factor; common dendritic progenitor; lineage commitment

Immune responses are orchestrated by a diverse group of functionally specialized, highly differentiated hematopoietic lineages. Surprisingly, such cellular heterogeneity can be established through the coordinated action of relatively few lineage-determining transcription factors. A striking demonstration of this principle is the direct conversion of terminally differentiated B lymphocytes into macrophages, where re-direction into the alternative fate requires only the expression of the transcription factors C/EBPα and C/EBPβ[1]. Similarly, forced expression of key transcription factors in progenitor stages can specify particular mature fates. For example, constitutive activation of the transcription factor *Notch1* in hematopoietic stem cells (HSCs) specifies commitment to the T cell lineage[2]. Inversely, deletion of *Notch1* redirects HSCs to B or natural killer (NK) cell fates[3,4].

Although cellular differentiation has been studied extensively in the immune system, the molecular mechanisms regulating dendritic cell (DC) development are underdeveloped relative to other lineages. DCs bridge two phases of the immune response: initial recognition of pathogens through pattern recognition receptors; and specific cell- and antibody-mediated clearance[5]. Despite the importance of these cells, their relative paucity and phenotypic similarity to other cells of the mononuclear phagocyte system (MPS) have confounded analysis. Moreover, DCs comprise several distinct subsets for which precise functions and interrelationships have been difficult to decipher. In this Review, we focus on advances in

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The authors have no conflicting financial interests.

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three areas that have helped to distinguish these cells from related lineages and to determine their function *in vivo*. First, we discuss the cellular intermediates and branch points of DC development from HSCs. Second, we review recent evidence establishing classical DCs as a distinct lineage among myeloid cells. Finally, we cover insights into the function of DC subsets gained from understanding the molecular basis for their diversification.

Lineage diversity in the MPS

Ralph Steinman and colleagues discovered DCs as a unique cell type with “stellate” or dendritic morphology in preparations of adherent splenocytes[6]. These classical (or conventional) DCs (cDCs) were designated as a distinct lineage based on their remarkable capacity for stimulating naïve T cells in a mixed lymphocyte reaction and their biochemical divergence from macrophages[7–9]. Subsequently, several cell types have been described with phenotypic and functional attributes resembling those of Steinman’s cDCs[10]. It has been difficult to determine the relatedness of these cell types based on cell surface markers and functional responses, as these attributes can overlap among myeloid cells and vary depending on cellular activation status and location within the body[11]. At present, four major cell types are generally categorized as DCs: cDCs, plasmacytoid DCs (pDCs), Langerhans cells and monocyte-derived DCs. We discuss these populations briefly and compare them to the functionally distinct macrophage lineage.

Classical dendritic cells (cDCs)

cDCs are highly phagocytic, specialized antigen-processing and -presenting cells. A key feature of cDCs is their short half-life (approximately 3–5 days) and continuous replacement from bone marrow (BM) precursors, a process dependent on the cytokine Flt3L (FMS-related tyrosine kinase 3 ligand)[12–15]. Importantly, these cells encounter and capture antigens in peripheral tissues and migrate via afferent lymphatic vessels to the T cell zones of secondary lymphoid organs to initiate adaptive immune responses[16] (Fig. 1). To accomplish these functions, cDCs exist in two distinct functional states[17,18]. Newly differentiated, immature cDCs exhibit high endocytic activity but display lower surface levels of major histocompatibility complex (MHC) class I and II proteins. Upon encounter with microbial products or inflammatory stimuli, cDCs undergo marked cytoplasmic reorganization, transporting peptide-MHC complexes to the cell surface and upregulating costimulatory molecules.

Heterogeneity in cDCs was first evidenced by the expression of CD8α on a small subset of thymic and splenic DCs; this remains the principal component for distinguishing cDC subsets[19,20]. In addition to CD8α^+ cDCs, a second and largely complementary subset can be distinguished in lymphoid organs by expression of CD4[21,22]. These two subsets may have evolved to provide distinct functions[23]. Early studies determined that CD8α^+ cDCs are highly efficient at cross-presenting exogenous antigens on MHC class I molecules to CD8$^+$ T cells[24,25]. In contrast, CD4$^+$ cDCs are generally considered to be poor cross-presenters *in vivo* but may be more efficient in priming CD4$^+$ T cells through MHC class II-restricted presentation[26,27].

Recent studies have identified two cDC subsets in peripheral non-lymphoid organs with analogous functions and developmental requirements[28–33]. In peripheral tissues, CD8α^+ cDC equivalents lack their namesake marker but are instead identified by expression of CD103 (integrin $\alpha_E\beta_7$)[31–34]. Similarly, CD4 is not expressed on the complementary peripheral subset, which can be instead identified by the high expression of CD11b. Here, we will consider peripheral CD103$^+$ and lymphoid-resident CD8α^+ cDCs as one unified subset (although there may be differences due to organ-specific milieu), and CD11b$^+$ and CD4$^+$ cDCs as a second, separate lineage[23,35].

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Human counterparts for CD8α^{+} and CD11b^{+} cDC subsets have been identified in lymphoid and non-lymphoid organs, underscoring the importance of DC lineage diversification across species[35–43]. CD8α^{+} cDC equivalents in humans, identified by their expression of XCR1, BDCA3 (CD141), and CLEC9A, are also able to cross-present antigens from dead or necrotic cells. In contrast, human BDCA1 (CD1c)$^{+}$ DCs lack CD8α^{+} cDC-specific functions and transcriptionally resemble murine CD11b^{+} cDCs[35,37].

Plasmacytoid dendritic cells (pDCs)

pDCs are a distinct lineage based on morphology, gene expression and ability to secrete high levels of type 1 interferon following viral encounter[44–47]. In contrast to the "dendritic" appearance of cDCs, pDCs exhibit a spherical shape characteristic of antibody-secreting plasma cells. By surface markers, pDCs are distinguished from cDCs by B220, Siglec-H, and Bst2 in mice and by BDCA2 (CD303) in humans[48–51]. Functionally, pDCs are not phagocytic and maintain a high rate of MHC class II turnover on their cell surface, rendering them inefficient at presenting exogenous antigens to CD4^{+} T cells[52]. pDCs are developmentally related to cDCs; both derive from common BM progenitors and require Flt3L for differentiation, though pDCs diverge from cDCs during maturation in the BM[53,54]. Moreover, following activation, pDCs can acquire some cDC characteristics such as dendritic morphology, an observation that formed the basis for their original designation as DC precursors[48].

Macrophages

Unlike DCs, macrophages are non-migratory, tissue-resident cells that are generally inefficient at antigen presentation. Instead, they possess high proteolytic and catabolic activity, which contributes to their ability to scavenge and ingest pathogens, dead cells and cellular debris[55]. At steady state, macrophages are considered to be anti-inflammatory, maintaining organ homeostasis in part by producing regulatory cytokines such as IL-10[56–58]. Under inflammatory conditions, macrophages can become "classically activated" and participate in the host response to pathogens[57].

While macrophages have been categorized into organ-specific subsets such as bone osteoclasts, liver Kupffer cells, splenic red-pulp macrophages and lung alveolar macrophages, these populations can function similarly upon activation and thus may not represent distinct lineages[57]. However, within each organ, tissue macrophage populations can be separated into CD11b^{hi}F4/80^{int} and CD11b^{int}F4/80^{hi} subsets[59]. This distinction reflects their derivation from two distinct hematopoietic sources: CD11b^{hi}F4/80^{int} macrophages originate from HSC-derived monocytes, while CD11b^{int}F4/80^{hi} macrophages develop from a yolk sac macrophage population emerging before definitive HSC development. These two macrophage populations display some differences in gene expression but are unified in their dependence on macrophage colony stimulating factor receptor (M-CSFR) and the transcription factor PU.1 for development[59].

Classically, Langerhans cells (LCs) have been viewed as a DC population in the epidermis[60–62]. However, they resemble tissue-resident macrophages—particularly, microglia—in several ways. LCs are developmentally dependent on M-CSFR signaling, express macrophage-specific markers such as F4/80, migrate poorly to lymph nodes in comparison to cDCs, constitutively secrete IL-10 and display an overall gene expression profile similar to macrophages[63–66]. Furthermore, microglia and LCs arise exclusively from embryonic macrophage and monocyte populations in the steady state and specifically require IL-34 for prenatal development[67–69]. A reconsidered view might therefore characterize LCs as a macrophage-related lineage rather than a cDC subset, although even this classification is somewhat blurred, as activated LCs exhibit increased migratory

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behavior similar to cDCs but unlike macrophages[62,64]. Indeed, gene expression analysis of resident and migratory LC populations reveals that migrated LCs acquire hallmarks of the cDC signature, including expression of the Flt3L receptor *Flt3*, *Ccr7* and the transcription factor *Zbtb46*[66].

Monocytes

Monocytes derive from BM progenitors, circulate in the blood and can differentiate into macrophages and DCs[70]. They can be separated into two subsets based on phenotype and function. The $Ly6C^{lo}CX3CR1^{hi}CCR2^{lo}$ subset, called "patrolling monocytes," migrates along the luminal surface of the vascular endothelium[71]. Close association with blood vessels allows for rapid recruitment to sites of infection, first serving to produce inflammatory mediators and then differentiating into alternatively activated macrophages to initiate tissue repair[70]. The second subset comprises $Ly6C^{hi}CX3CR1^{lo}CCR2^{hi}$ cells that have been called "inflammatory monocytes." These cells are recruited to sites of infection with slightly delayed kinetics and differentiate into TNF and inducible nitric oxide synthase (iNOS)-producing "inflammatory DCs" (Tip-DCs). Tip-DCs, which were initially so named based on their dendritic appearance and expression of CD11c, are thought to represent the main inflammatory cell type during infection[72,73]. In humans, two subsets of monocytes with parallel functions are distinguished by the expression of CD14 ($Ly6C^{hi}$ equivalent) and CD16 ($Ly6C^{lo}$ equivalent)[70,72].

The induction of DC differentiation *in vitro* from human and mouse peripheral monocytes by granulocyte macrophage colony stimulating factor (GM-CSF) first suggested that monocytes may be an important reservoir for DC development[74,75]. Like cDCs, GM-CSF-derived DCs upregulate CD11c and MHC class II expression and efficiently stimulate naïve T cells. Although this *in vitro* system has been widely used, the *in vivo* equivalent of the GM-CSF-derived DC has been difficult to identify. Tip-DCs, the initial candidate[27,73,76], are more likely to represent activated monocytes rather than cDCs based on gene expression and on their normal development in GM-CSFR-deficient mice[11,77–79]. $DC\text{-}SIGN^{+}MHCII^{hi}$ cells induced following microbial stimulation, another candidate for the *in vivo* equivalent of GM-CSF-derived DCs[80], are in fact dependent on Flt3L signaling and possibly represent activated cDCs that develop independently of monocytes[78]. Finally, recent studies have suggested that monocytes transferred following depletion of host DC populations can give rise to DCs in the intestine[58,81,82]. These donor-derived cells seem to resemble bona fide cDCs by function and phenotype, but their GM-CSF dependence and gene expression patterns have not been reported[58,83].

DCs develop from progressively restricted BM progenitors

DCs in lymphoid organs and peripheral tissues are short-lived and continuously repopulated from cells derived from the HSC[14]. Adoptive transfer studies initially suggested that DCs could arise from either lymphoid- or myeloid-restricted progenitors[84]. While the precise contribution of common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) to the DC pool is still debated, a recent fate-mapping study determined that ~10% of DCs in lymphoid organs develop from interleukin 7 receptor (IL-7R)-expressing CLPs, suggesting that ~90% derive from CMPs[85]. Macrophage-dendritic cell progenitors (MDPs), the first precursor population downstream of the CMP that retains DC potential[86,87], give rise to pDCs, cDCs, monocytes and macrophages, but not neutrophils or other myeloid lineages. However, whether MDPs truly represent a clonal source of both M-CSF-dependent macrophages and Flt3L-dependent DCs is still under investigation (K. Shortman, personal communication). Subsequently, a common dendritic cell progenitor (CDP)[53,54] was identified that retains only cDC and pDC potential[53,54]. Like MDPs, CDPs express high

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levels of M-CSFR and Flt3 but lower levels of the stem cell factor receptor (c-Kit)[87]. At a clonal level, CDPs are able to generate all DC subsets[53] and appear to represent the immediate precursor of pre-cDCs, which are cDC-restricted but immature[88,89]. Pre-cDCs seed lymphoid and non-lymphoid tissues via the blood and then complete differentiation into either CD8α$^+$ or CD11b$^+$ cDCs (Fig. 1). In contrast, pDCs mature in the BM and emigrate through the blood to secondary lymphoid organs. Unlike fully differentiated CCR9$^+$ pDCs, immature CCR9$^-$ pDCs can differentiate into cDC subsets *in vitro* and *in vivo*[90], suggesting that pDC development can be redirected until the terminal stage of maturation[91].

Flt3L and M-CSF are the two major regulators of DC and macrophage development, respectively[92]. Flt3 expression is maintained throughout DC development and on terminally differentiated DCs but not on macrophages[66,92]. Loss of Flt3L, Flt3 or its downstream signaling molecule STAT3 greatly reduces DC numbers *in vivo*[12,13,15,93]. Inversely, forced expression of Flt3 on Flt3$^-$ progenitors rescues their ability to give rise to DCs *in vivo*[94]. Flt3 signaling serves to promote CDP proliferation as well as homeostatic expansion of DCs[15]. In contrast, M-CSFR is maintained on mature macrophages and progressively downregulated on DCs[66,92]. Loss of M-CSFR, but not Flt3, specifically impairs monocyte and macrophage development[95]. However, the dichotomy between Flt3 and M-CSFR is an oversimplification, as other cytokines likely play a role in development. For example, the combined loss of GM-CSF and Flt3L generates a more severe reduction in DC development than loss of Flt3L alone[96]. Similarly, loss of lymphotoxin signaling leads to the selective reduction of DCs in the spleen and intestine but not in other peripheral tissues[97,98]. Taken together, these observations suggest that distinct combinatorial niches or pathways may operate independently for the generation of DCs or macrophages *in vivo*.

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Surface marker limitations in distinguishing myeloid lineages

For some time, CD11c was the main surrogate marker for DC identification; its locus has been modified to express fluorescent reporters, Cre recombinase, or diphtheria toxin receptor (DTR) to allow tracking, deletion or inducible depletion of DCs *in vivo*[99–101]. However, the interpretation of studies based on these models has been confounded by *CD11c* expression in other lineages[102,103]. For example, administration of diphtheria toxin A (DTA) in *CD11c*-DTR mice depletes cDCs and pDCs along with tissue-resident, marginal zone and metallophilic macrophages, as well as NK cells, NKT cells and some CD11c$^+$ B and T cells[65,104,105]. To date, no single surface marker has uniquely distinguished DCs from other myeloid or lymphoid lineages.

The identification of subsets within the cDC lineage using surface markers can also be problematic. For example, while CD103 is commonly used to identify peripheral CD8α$^+$ cDCs, it is also expressed on CD11b$^+$ cDCs in the intestine and lung[33,81]. Furthermore, CD103 is dynamically regulated by a number of cytokines, including GM-CSF, IL-3 and TGF-β[106–108]. This has generated some debate as to whether GM-CSF regulates the development of peripheral CD103$^+$ cDCs and intestinal CD11b$^+$ cDCs or merely controls the expression of CD103[79,81,106,109]. One study reported that CD103$^+$CD11b$^-$ cDCs develop in all organs but express lower levels of CD103 in the absence of GM-CSFR[106]. In contrast, another study reported an absence of CD103$^+$ cDCs in the peripheral tissues of GM-CSFR-deficient mice[79]. Experiments using mixed chimeras revealed that DC progenitor development is unaffected; GM-CSFR$^{-/-}$ CD103$^+$ cDCs can develop but exhibit reduced survival relative to wild-type cDCs[79].

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Additional loci have been targeted to express fluorescent proteins to define myeloid lineages *in vivo*, including *Lysm*, *Csf1r*, *Cx3cr1*, and *CD11b*, but these are also dynamically

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regulated and expressed outside the DC lineage[65]. Such difficulties have prompted suggestions that identifying bona fide cDC populations may require simultaneous analysis of surface phenotype, cellular derivation, function and anatomical location[11], or even that the effort may be inherently futile[65].

Refining lineage relationships through transcriptional programs

The Immunological Genome Project (ImmGen) expression database includes more than 50 macrophage and dendritic cell subsets, making possible an unbiased assessment of genetic relationships among cell types[66,110,111] (Gautier E.H. and Randolph G.J., in press). Using principal component analysis (PCA), correlated variables from these high-dimensional datasets are expressed in fewer uncorrelated principal components (PCs) representing the variance among samples. In a PCA using several macrophage and DC samples, more than two-thirds of the gene expression variation is captured in three PCs, which segregate cell types by anatomic location (Fig. 2a) and by lineage independent of location (Fig. 2b). When genes are ordered by their relative "weight," or loading, in PC3, the most positive loadings correspond to genes such as *Flt3* and *Xcr1*, known to be involved in DC but not macrophage development and function; the most negative loadings correspond to *C1q* and *Ctsd*, which function specifically in macrophages (Fig. 2b). In extensive examinations of DC and macrophage populations, several groups have observed similar segregation by organ and lineage in PCAs[66,111] (Gautier E.H. and Randolph G.J., in press). Notably, PCAs incorporating LCs have consistently positioned these cells closer to macrophages[66] or, specifically, to microglia (X.W., unpublished) than to cDCs along the lineage-associated principal component.

These analyses suggest that approaches beyond surface markers may be valuable in separating lineage from the effects of maturity and anatomic location. Recently, the transcription factor *Zbtb46* was identified as a marker specifically expressed by cDCs in lymphoid and non-lymphoid tissues but not by other myeloid or lymphoid cell types[77,78]. Using DTR-based ablation or a GFP reporter, these studies demonstrate that a transcription factor-based approach can be useful in distinguishing DCs from macrophages (Fig. 3). In peripheral organs such as the lung, *Zbtb46* is uniformly expressed in $CD11c^+CD103^+CD11b^-$ and $CD11c^+CD103^+CD11b^+$ cDC populations, confirming their cDC classification, but not in $CD11c^+SSC^{hi}CD103^-CD11b^{lo/-}$ macrophages. In addition, *Zbtb46* is expressed in a fraction of $CD11c^+CD103^-CD11b^+$ cells, demonstrating previously unappreciated heterogeneity within this population. In contrast, Tip-DCs and LCs show little to no expression of *Zbtb46*, arguing for a macrophage rather than DC identity. For rare cells and cells difficult to identify using FACS-based methods, *Zbtb46*-GFP mice permit direct GFP visualization in tissue sections[77] or with intravital two-photon microscopy (Z. Schulman and M.C. Nussenzweig, personal communication). Visualization of $CD169^+$ subcapsular sinus macrophages (SSMs) revealed that a subset of these cells expresses *Zbtb46*. It is still unclear whether this heterogeneity is related to the recently described transfer of CD169 between SSMs and innate lymphocytes, further motivating the development of additional transcription factor-based lineage-tracking approaches[112].

An unanticipated utility of the *Zbtb46*-GFP reporter emerged from its heterogeneous expression within previously defined DC progenitor populations[77]. Within the CDP and pre-cDC gates, *Zbtb46* expression identifies a subset of cells with completely extinguished pDC potential, whereas previous pre-cDC demarcations retain appreciable pDC output[77,89]. Specifically, pre-cDCs are subdivided into four populations based on the expression of *Zbtb46*-GFP and Siglec-H. *Zbtb46*-GFP^+ cells generate cDCs but not pDCs regardless of Siglec-H expression. In contrast, $SiglecH^+$*Zbtb46*-GFP^- cells are not committed to the pDC lineage and can induce *Zbtb46* expression and generate cDCs. Hence, expression of Siglec-

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H on pre-cDCs may identify a population overlapping with recently described CCR9$^-$ pDC-like precursors[90]. Combining *Zbtb46*-GFP with additional markers may further resolve DC progenitor potential and, perhaps, reveal the basis for divergence of CD8α$^+$ and CD11b$^+$ cDCs.

More generally, transcription factor-based reporters may help to distinguish other mature myeloid lineages and clarify lineage potentials within currently defined progenitor stages. While *Zbtb46* expression is positively correlated with the lineage-segregating component score by PCA, *Mafb* expression shows an inverse pattern (Fig. 2c). Indeed, both genes are key elements of the core transcriptional signature of cDCs and macrophages, respectively[66] (Gautier E.H. and Randolph G.J., in press). Thus, *Mafb* reporter mice may more accurately identify stages of macrophage commitment[113] (Fig. 3). Similarly, pDC development may be clarified by reporters for *E2-2*[114,115] and the pDC-specific factors *Spib* and *Duxbl*[116], while an *Irf8* reporter could help distinguish DC-committed progenitors from MDPs[117] (Fig. 3).

Transcriptional factor networks regulate DC subset heterogeneity

Recent reviews of DC development have distinguished between transcription factors acting generally in immune cell development and those acting specifically within DC lineages[91,118]. Factors of the first kind include Ikaros, PU.1, and Gfi1, which regulate genes required for early hematopoiesis such as *Flt3, Il7r, and Stat3*[119–124]. Whether these factors also exert DC-restricted actions has not been examined by lineage-specific conditional deletion. Here, we focus on factors of the second kind, which regulate development of DC subsets and allow for their functional analysis.

CD8α$^+$ cDC transcription factors

DC development is heavily affected by the transcription factor IRF8, one of nine IRF family members. IRF8 deficiency causes complete loss of pDCs and CD8α$^+$ cDCs[125]. In the setting of competitive BM reconstitution, IRF8 is also important for development of CD11b$^+$ cDCs[117]. The reduction of all cDC subsets in the absence of IRF8 indicates that this factor acts very early in DC-committed progenitors (Fig. 4a); accordingly, CDPs are greatly reduced in the absence of IRF8, while an associated increase in neutrophil precursors is observed[117]. Among potential gene targets of IRF8 that may be key in fate restriction, several are known to regulate DC development[117] (Fig. 4a). Id2 is induced *in vitro* by GM-CSF and required *in vivo* for development of CD8α$^+$ cDCs but not other subsets[126]. Bcl6 is induced at the pre-cDC stage and required for development of CD11b$^+$ and CD8α$^+$ cDCs but not pDCs[127]. Klf4 and Bach2 are highly expressed in DC progenitors and mature DCs[110]; loss of Klf4 causes a moderate reduction in CD11b$^+$ cDCs[128], but no role for Bach2 in DCs has yet been reported.

Unlike *Irf8*, the AP-1 factor *Batf3* is expressed in both CD11b$^+$ and CD8α$^+$ cDCs but is required for normal development of only the latter subset[33,129]. Batf3 heterodimerizes with Jun paralogs but lacks the carboxy-terminal transcriptional activation domain found in Fos, the classical AP-1 partner of Jun[130]. Batf3 does not influence the development of progenitors such as CDPs or pre-cDCs but instead acts in the final stages of CD8α$^+$ cDC development[33]. Interestingly, CD8α$^+$ cDCs can be found in skin draining lymph nodes of C57BL/6 *Batf3*$^{-/-}$ mice at steady state and in additional organs following administration of IL-12[106,131]. This compensatory development relies on the related factors Batf and Batf2 to form AP-1-IRF DNA complexes[131].

A recent analysis of *Id2*-GFP reporter mice showed the sequential requirement of IRF8, Id2 and Batf3 in the development of CD8α$^+$ cDCs[107] (Fig. 4a). This study confirmed that Batf3 acts only in the final stage of CD8α$^+$ cDC maturation, during which CD103 is induced,

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whereas IRF8 is required for development for all *Id2*-expressing cDC progenitors. Recently, Nfil3 was identified as another transcription factor required for CD8α$^+$ cDC development[132]. *Batf3* expression is reduced in *Nfil3*$^{-/-}$ DC progenitors and Batf3 overexpression bypasses the requirement for Nfil3 in CD8α$^+$ cDCs, suggesting that Nfil3 may act upstream of Batf3; however, additional analysis is required to place this factor in the context of IRF8 and Id2.

Orthologous factors may act in the specification and commitment of human and murine DC subsets. For example, human BDCA3$^+$ and BDCA1$^+$ cDCs, the equivalents of murine CD8α$^+$ and CD11b$^+$ cDCs respectively[133], show IRF8 dependence[134]. A patient presenting with severe opportunistic infections was found to carry an IRF8 K108E mutation which impairs DNA binding and transactivation[134]. As in mice, deficiency of IRF8 resulted in the loss of monocytes, pDCs and both cDC subsets, suggesting that this factor also acts at an early stage of DC development in humans. Human BDCA3$^+$ cDCs also show a dependence on BATF3; knockdown of BATF3 in human cord blood progenitors specifically leads to the diminished development of CLEC9A$^+$BDCA3$^+$ cDCs *in vitro*[133].

CD11b$^+$ cDC transcription factors

The first transcription factor observed to regulate the development of a specific DC subset was the NF-κB family member RelB[135,136]. Mice deficient in RelB have a marked reduction of CD11b$^+$ cDCs due to a cell-intrinsic defect[136]. Similarly, a requirement for IRF4 has been reported for CD11b$^+$ cDC development[137]. This factor interacts with PU.1 and can either activate or repress gene expression[138]. It is not yet known how IRF4 and RelB act to regulate CD11b$^+$ cDCs or what signal is responsible for their induction in developing progenitors. Moreover, additional analysis of these factors using a more complete array of surface markers and reporter strains is needed to explain some previous observations. For example, loss of IRF4 produces an increase in CD11c$^+$CD4$^-$CD8α$^-$ cells, but the identity of these cells has not been evaluated[137].

The recognition that canonical Notch2 signaling regulates CD11b$^+$ cDC development represents an important recent advance[98,101]. Mice deficient in RBP-J, a mediator of the Notch signaling pathway, show a 50% reduction specifically in splenic CD11b$^+$ cDCs[101]. It has since been recognized that splenic CD11b$^+$ cDCs comprise two subsets distinguished by CX3CR1 and ESAM expression; Notch2, but not other Notch receptors, is required specifically for development of CX3CR1^{lo}ESAM^{hi} cells[98]. While both subsets are dependent on Flt3 signaling, the Notch2-dependent cells selectively require lymphotoxin-β receptor (LTβR) for development. Because LTβR is required for the homeostasis of splenic DC subsets[97], Notch2 might act by promoting DC precursor interaction with marginal zone cells producing LTα_1 β_2. Deficiency of Notch2 (or, presumably, LTβR) does not affect migratory DCs in tissues other than the intestine, where all CD11b$^+$CD103$^+$ cDCs are absent. These results confirm that intestinal CD11b$^+$CD103$^+$ cDCs are not related to Batf3-dependent CD103$^+$ cDCs present in other peripheral tissues but rather represent an intestinal equivalent of CD11b$^+$ lymphoid cDCs. Further, ESAM^{lo} DCs are thought to derive from the MDP independently of the pre-cDC[98], although it is possible that ESAM^{hi} and ESAM^{lo} CD11b$^+$ cDCs arise from a common progenitor in which the terminal fate is determined at the anatomic site of maturation. Finally, Notch2 may also regulate maturation of CD8α$^+$ cDCs, but the extent of this effect—and how much it may coincide with the role of this factor in CD11b$^+$ cDCs—requires additional analysis[98].

pDC transcription factors

The transcription factor E2-2 is specifically required for pDC development in both mice and humans[114]. E2-2 is a member of the class I basic helix-loop-helix (bHLH) family along with

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three other factors: E12, E47, and HEB [139]. These E proteins form dimers with each other that bind conserved E-box DNA motifs, an action that can be interrupted when E proteins dimerize instead with proteins of the inhibitor of differentiation (Id) HLH family such as Id2. Indeed, in the pre-cDC, E2-2 inhibition by Id2 may divert cells away from pDC differentiation and re-direct them along the cDC pathway. Overexpression of Id2 inhibits *in vitro* pDC development[140], while conditional deletion of *E2-2* in mature pDCs results in the re-expression of many genes not associated with pDC identity, including *Id2* itself[115]. Further, pDCs are increased in *Id2*$^{-/-}$ mice[126], although more detailed analysis using pDC-specific markers may be warranted. Direct transcriptional targets of E2-2 in pDCs, identified by chromatin immunoprecipitation with microarray analysis (ChIP-on-chip), include the transcription factor genes *Spib*, *Irf8* and *Bcl11a* as well as several genes associated with pDC function such as *Tlr7, Tlr9* and *Bdca2*[114,115]. In fact, both Spi-B and Bcl11a have now been hypothesized to play roles in pDC development[115,116].

Functions of DC subsets determined by genetic models

Recently developed genetic models, including transcription factor-deficient mice, have helped advance the understanding of DC function (Fig. 4b). To address the imperfect separation of cDC and macrophage functions using CD11c-based systems[103,141], *CD11c*-DTR and *Zbtb46*-DTR mice were compared in the context of *Toxoplasma gondii* infection and tumor growth[78]. DTA-mediated depletion in *CD11c*-DTR mice leads to significantly greater impairment of immune responses in both models in comparison to *Zbtb46*-DTR mice: increasing pathogen burden, diminishing antigen-specific T cell priming and allowing greater tumor growth. These results argue that populations deleted in *CD11c*-DTR mice but not *Zbtb46*-DTR mice actively contribute to immune defenses. Thus, assessment of cDC-specific functions *in vivo* may benefit from re-evaluation using the more stringent *Zbtb46*-DTR system.

Similar insights into the role of the CD8α$^+$ cDC were gained using mice deficient in Batf3 or IRF8. Although IRF8-deficient mice previously implicated CD8α$^+$ cDCs as a source of IL-12 during infection by *T. gondii*[142], these mice harbor additional changes in other myeloid lineages, B cell development and expression of IFN-γ-inducible genes, all of which may contribute to altered immune responses[143,144]. Because *Batf3*$^{-/-}$ mice show a more selective defect restricted to the CD8α$^+$ cDC, studies using these mice helped confirm that the CD8α$^+$ cDC is the critical source of IL-12 limiting early *T. gondii* infection[129,145]. The capacity for robust IL-12 production, which may be facilitated by expression of innate sensors such as TLR11, demonstrates that cross-presentation capacity is not the only consequence of CD8α$^+$ cDC specialization[145]. *Batf3*$^{-/-}$ mice have also helped to clarify the role of CD8α$^+$ cDCs as an obligate entry point in promoting the early spread of *Listeria monocytogenes* infection from the marginal zone to the splenic lymphoid areas in a blood-borne infection model[146,147]. Furthermore, analysis of *Batf3*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice together revealed that CD8α$^+$ cDCs require type I interferon signaling to promote their anti-tumor activity[148,149]. This finding agrees with recent studies indicating that cross-presentation by CD8α$^+$ cDCs may require maturation induced by GM-CSF or TLR activation[108,150].

Analysis of *Batf3*$^{-/-}$ mice has also led to the exclusion of some proposed actions of CD8α$^+$ cDCs. For example, CD8α$^+$ cDCs are apparently not essential for the development of regulatory T cells (T_{regs})[129,151]. Likewise, CD103$^+$ cDCs are dispensable for the induction of experimental autoimmune encephalitis (EAE)[106], contrary to past suggestions[109], and are not required for DSS-induced colitis or contact hypersensitivity[33], clarifying previous conflicting results[28,152]. And although CD8α$^+$ cDCs are required for responses to West Nile virus[129,153], herpes simplex virus[154] and mouse cytomegalovirus (MCMV)[155], they are not required for the inflation of immunodominant MCMV-specific T cells during latency[155].

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Lastly, priming of $CD8^+$ T cells to some viruses such as lymphocytic choriomeningitis virus (LCMV) may occur normally in the absence of $CD8\alpha^+$ cDCs (A. Pinto and M. Diamond, personal communication), although reports of such negative results are still awaiting publication.

In contrast to $CD8\alpha^+$ cDCs, the unique function of the $CD11b^+$ cDC remains to be determined, in part due to lack of genetic models that selectively delete this subset. Progress along these lines has been made with conditional deletion of *Notch2* using *CD11c*-Cre, which causes a defect in $CD4^+$ T cell priming and IL-17 production by intestinal $CD4^+$ T cells[98]. These findings are consistent with previous reports that $CD11b^+$ cDCs can support the priming of T_H17 cells *in vitro*[56] and that $CD11b^+$ cDCs produce IL-23 in response to TLR5 signaling[156]. Thus, cDC subsets may specialize in selectively priming $CD4^+$ T cells along different developmental pathways (Fig. 4b). This interpretation is consistent with a scheme in which $CD11b^+$ cDCs stimulate innate immune lymphocytes to produce IL-22 during infection, an important pathway for resistance against attaching and effacing bacteria such as *Citrobacter rodentium*[157,158].

Finally, genetic models have contributed to definitive attribution of pDC function. Using a mouse in which DTR is expressed under the control of the pDC-specific gene *Bdca2*, it was found that pDCs are required for the early production of type I interferon following viral challenge[159]. Deletion of pDCs does not dramatically alter viral burden or T cell priming following viral challenge, eliminating antigen presentation as major function of pDCs. Rather, pDCs induce early activation of NK cells and promote the survival of $CD8^+$ T cells following their expansion. These findings were confirmed using a model in which E2-2 is specifically deleted by *CD11c*-Cre, where it was shown that pDCs are particularly critical for controlling chronic viral infection. In this setting, the observed effect on T cell survival is mediated directly by pDC-dependent interferon production and is not a result of antigen presentation[160].

Concluding remarks

Advances in FACS-based cell isolation techniques have made possible the purification of committed DC progenitor populations, the creation of microarray databases and the identification of transcription factors that have led to animal models in which DC development can be manipulated *in vivo*. The resulting picture illustrates that DCs are in fact a lineage distinct from macrophages performing unique and non-redundant functions. Future work with genetic depletion models and with a range of pathogens is likely to form the basis for a more comprehensive understanding of the functions of specific DC subsets.

At the molecular level, recent work has begun to clarify the transcriptional mechanisms controlling DC development. These include identification of the role of E2-2, Batf3, Nfil3 and Notch2 in DC subset commitment and in-depth analysis of IRF8 and Id2, which have provided a hierarchical structure for their action in developing progenitors. However, many key questions still remain. The critical divergence between macrophages and DCs remains unexplained. Similarly, the basis for the bifurcation of $CD11b^+$ and $CD8\alpha^+$ cDC lineages remains to be defined. As in T or B cells, a full understanding of the stage-specific actions of transcription factors and their gene targets will benefit from unbiased genome-wide approaches such as microarray analysis, ChIP-seq and newer modalities. Ultimately, such work on DCs, particularly in humans, could have direct bearing on approaches to improve vaccine design for important infections and malignancies.

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METHODS

At least two replicate microarray datasets for each subset[110] were pre-processed by DNASTAR ArrayStar (version 4) using global median normalization and robust multichip average (RMA) summarization, then replicates grouped by sample. Log-transformed expression values were exported in tabular format, re-imported into R (version 2.13.2), mean-centered by gene, root mean square (RMS)-scaled by sample, transposed, and subjected to principal component analysis computed by singular value decomposition (without further centering or scaling). Scores were plotted in R or exported and plotted against log-transformed expression values in GraphPad Prism (version 5).

Acknowledgments

We thank the ImmGen Consortium for use of the ImmGen database[110] and the Alvin J. Siteman Cancer Center at Washington University in St. Louis for use of the Center for Biomedical Informatics and Multiplex Gene Analysis Genechip Core Facility. This work was supported by the Howard Hughes Medical Institute, National Institutes of Health (AI076427-02), and Department of Defense (W81XWH-09-1-0185; to K.M. Murphy); the American Heart Association (12PRE8610005; to A.T. Satpathy); and the German Research Foundation (AL 1038/1-1; to J.C. Albring). The Alvin J. Siteman Cancer Center is supported in part by National Cancer Institute Cancer Center Support Grant #P30 CA91842.

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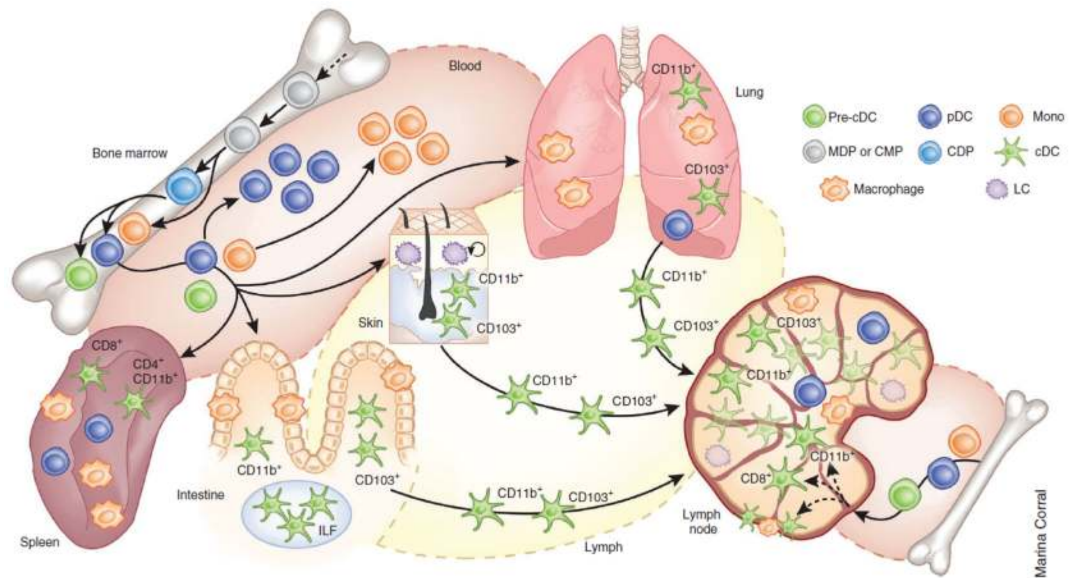

Figure 1. Development and migration of mononuclear phagocyte lineages in the steady state

Classical DCs (cDCs), plasmacytoid DCs (pDCs) and monocytes (Mono) derive from bone marrow (BM) progenitors. Macrophage-DC progenitors (MDPs) give rise to common dendritic cell progenitors (CDPs) and monocytes. CDPs differentiate into pDCs or committed precursors for cDCs (pre-cDCs). Pre-cDCs, pDCs, and monocytes transit through the blood and seed peripheral organs, where pre-cDCs complete their differentiation into CD8$^+$/CD103$^+$ or CD4$^+$/CD11b$^+$ cDCs. Monocytes can migrate into tissues and differentiate into macrophages. In the intestine, cDCs and macrophages populate the villi; cDCs are also present in intestinal lymphoid follicles (ILFs). In the skin, dermal DCs consist of both CD11b$^+$ and CD103$^+$ cDC subsets. Langerhans cells (LCs) populate the epidermis and self-renew locally. Macrophages, pDCs and both cDC subsets reside in the lung. A hallmark characteristic of cDCs is their ability to migrate upon antigen encounter from tissues to draining lymph nodes to prime T cell responses. In contrast, macrophages largely remain at the site of differentiation.

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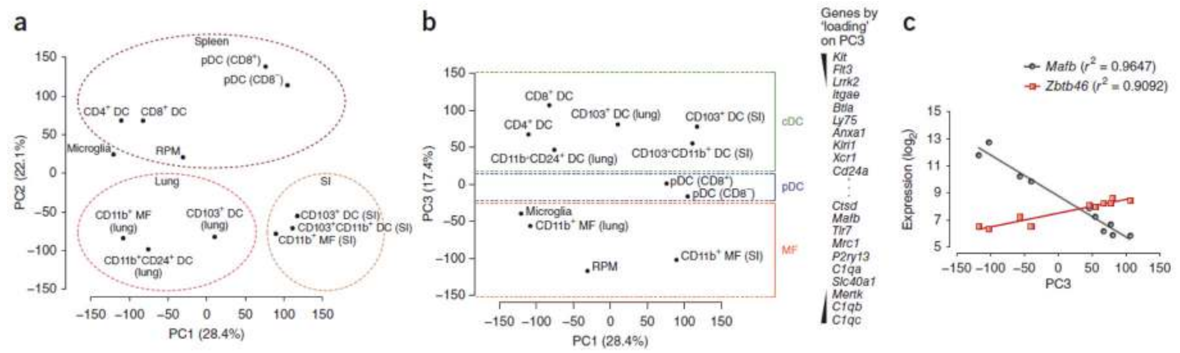

Figure 2. Global relationships between DC and macrophage subsets

Principal component analysis (PCA) of mature macrophage and DC subsets is shown. Each circle represents at least two microarray replicates. (**a,b**) Samples segregate by organ (PC1 and PC2) and by lineage (PC3). Genes with the greatest positive and negative loadings in PC3 reflect their DC- or macrophage-specific expression, respectively. Values in parentheses indicate proportion of variance explained. (**c**) Gene expression levels of *Mafb* and *Zbtb46* in cDC and macrophage subsets are compared against PC3 scores from **(b)**. MF, macrophage; PC, principal component; RPM, red pulp macrophage; SI, small intestine.

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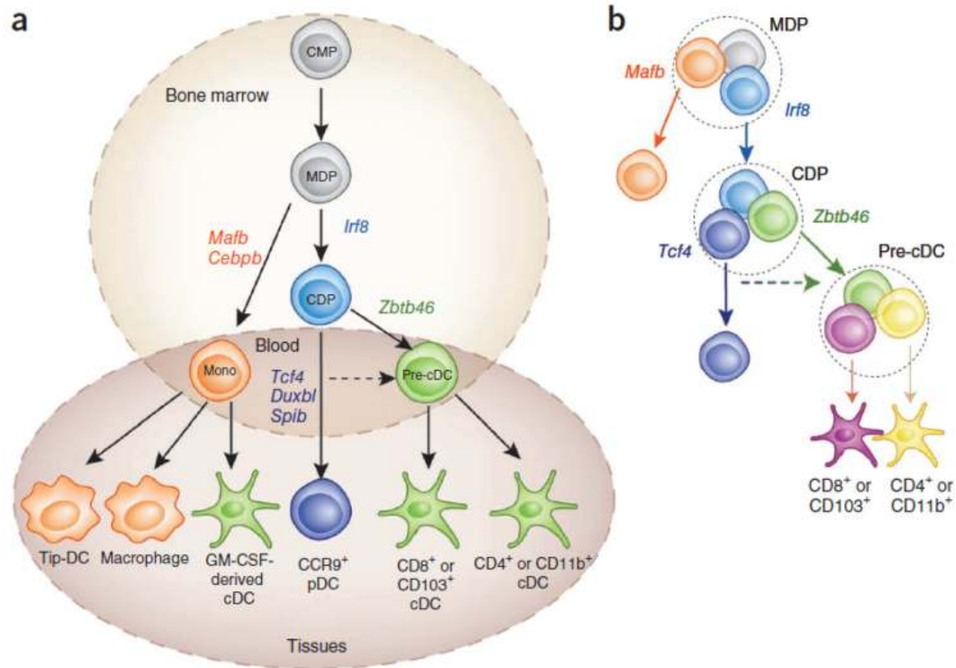

Figure 3. Distinguishing myeloid populations with lineage-specific transcription factors

(**a**) Lineage-specific transcription factor expression represents an alternative to surface marker-based methods for accurately identifying myeloid cell types. Lineage- or stage-specific transcription factors are indicated in colors corresponding to cell types in which they are uniquely expressed. For example, *E2-2* expression distinguishes the pDC lineage while *Zbtb46* distinguishes cDCs. (**b**) Shown is the theoretical heterogeneity within progenitor stages as defined by FACS (dashed circles). For example, cells characterized as CDPs using cell surface markers include a subpopulation of cells already committed to the cDC lineage, which are identified by expression of *Zbtb46*.

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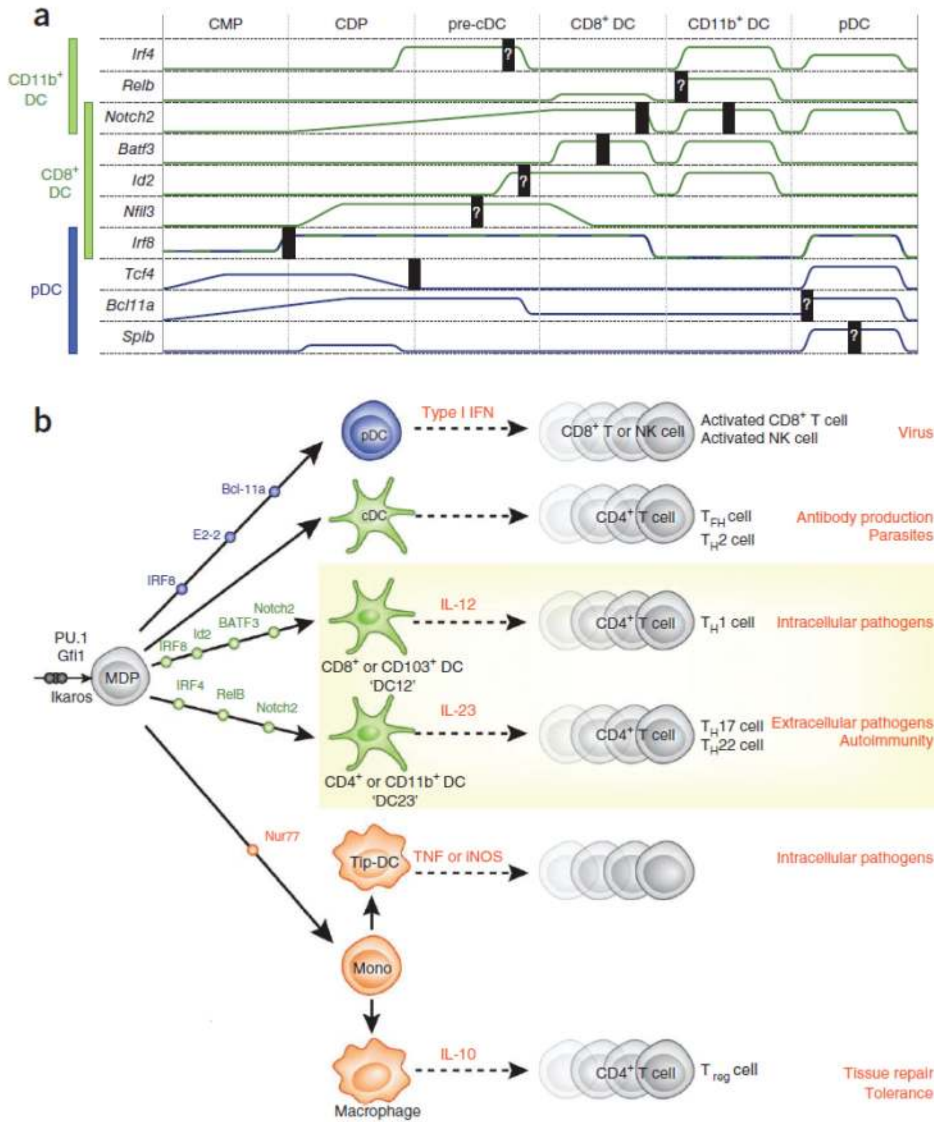

Figure 4. Stage-specific expression of transcription factors controlling DC development and specialization

(**a**) Shown are approximate mRNA expression levels[77,78,110] of selected transcription factors regulating DC development. Factors are grouped by the lineage in which they are required. Vertical bars indicate the stage at which each factor is essential for development. (**b**) Shown are sequential transcription factor requirements during development. Analysis of models deficient in these factors suggests that an important consequence of subset specialization is the ability to respond differentially to pathogen challenge through the secretion of specific cytokines. This specificity closely resembles the cytokine requirements for CD4^{+} T helper (T_H) subset differentiation, highlighting that DC responses are a key determinant of the resulting adaptive immune response. As in T cells, DCs may be better characterized by function rather than surface marker expression, i.e. CD8^{+} cDCs as “DC12” cells.