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Defining dendritic cells

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Dendritic cells (DCs) are versatile controllers of the immune system, best known for their potent ability to initiate adaptive immunity. Traditionally, DCs have been defined on the basis of cell morphology, expression of specific markers and select functional attributes such as the ability to migrate to T cell areas of secondary lymphoid organs and activate T lymphocytes. However, such properties are not qualitative and often change in conditions of inflammation or infection. Phenotypic-based and function-based definitions can therefore lead to difficulties in cell identification. Here we review other approaches to try and solve questions of DC lineage attribution with an emphasis on recent insights arising from our increased understanding of DC ontogeny and differentiation.

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What is in a name?

DCs were originally identified by Steinman and Cohn in mouse spleen on the basis of their unique morphology, which distinguished them from macrophages [1]. They were subsequently found to be the most potent stimulators of the mixed lymphocyte reaction [2], setting the foundation for decades of research demonstrating the importance of DCs in initiating adaptive immune responses. The name dendritic cell has become synonymous with motile cells of stellate morphology, expressing high levels of major histocompatibility complex class II molecules and the integrin CD11c [3,4], distinguished by their ability to migrate from non-lymphoid to lymphoid organs and their superior capacity to stimulate T lymphocytes [5–7]. This has been subsumed into the notion that DCs can be defined by their ability to migrate to secondary lymphoid tissues and prime T cells. This definition is useful but excludes the possibility that, in some instances, T cell priming may be carried out by monocytes or macrophages. It also glosses over the fact that DCs are a heterogeneous family of cells, subsets of which may in fact function to suppress rather than stimulate immune responses [8]. Finally, it ignores the point that, in some situations, DCs orchestrate innate immune responses independently of T cell activation or migration to secondary lymphoid organs [9•,10••,11,12••,13••]. These anomalies, allied to the lack of unique phenotypic markers that allow for unambiguous distinction of DCs from monocytes and macrophages, have led some to question the existence of DCs as an independent cell type with unique functional properties [14–16]. So, how can we circumvent these issues and define DCs other than by phenotype or function? Recent efforts to characterize DC precursors in mouse and human, hand-in-hand with parallel studies on the ontogeny of macrophages and monocytes [17,18••,19•,20••,21••,22•,23••,24,25], have suggested that DCs can be grouped together based on common descendence from a committed hematopoietic progenitor. Such an ontogenetic perspective allows for definition of DCs as a discrete hematopoietic lineage independently of cell phenotype or function, thereby permitting the unfettered exploration of DC roles in immunity and homeostasis [26].

Ontogeny of DCs

The classic model of DC development is primarily derived from mouse studies. A bipotent progenitor in the bone marrow, called macrophage and DC precursor (MDP), gives rise to DCs and monocytes [27–29]. MDPs further differentiate into common DC precursors (CDPs) restricted to the generation of plasmacytoid DCs (pDCs) and conventional DCs (cDCs) [30,31]. While pDCs terminally differentiate in the bone marrow [32], so called pre-DCs exit the bone marrow and migrate through the blood to lymphoid and non-lymphoid organs, where they terminally differentiate into cDCs, including the $CD8\alpha^+$/$CD103^+$ and $CD11b^+$ subsets [33,34]. Analogous to the CDP, a common monocyte progenitor (cMoP) has recently been identified that is downstream of MDPs and gives rise to monocytes but not DCs [18••].

Mouse MDPs express CX3CR1 and were originally identified for their ability to generate DCs and monocytes *in vitro*, as well as after transfer into mice [27–29]. Although evidence for MDP bi-potentiality was provided in those early studies [27], it has been put in question more recently [22•]. Additionally, 'MDP' populations now appear to exhibit substantial granulocyte potential [22•], which was not observed in the earlier studies

[18••,27] or in CX3CR1 fate mapping experiments [24]. Therefore, the existence of a bi-potential progenitor for DCs and monocytes has become a subject of contention. Added to this, CDPs, the presumed downstream developmental intermediate between MDPs and DCs, can produce pDCs [30,31]. By contrast, MDPs exhibit pDC potential in some [18••,29] but not other [27,28] studies. While these inconsistencies might be explained by disparities in cell purity after isolation they also indicate that MDPs might not always be developmental precursors of pDCs. Indeed, tracking of bar-coded progenitors transferred into irradiated mice indicates that lineage divergence among myeloid cell types might occur as early as a stage upstream of MDPs known as the lymphoid-primed multipotent progenitor (LMPP) [20••].

Mouse MDPs and CDPs exhibit substantial phenotypic overlap [29]. They both lack lineage specifying markers, express CD115 and CD135 in addition to CX3CR1 and can only be distinguished by the fact that CDPs express lower levels of CD117 (c-kit) than MDPs [27–31]. We have recently demonstrated that DNGR-1 (encoded by the *Clec9a* gene and also known as CLEC9A) marks cells resembling CDPs but not MDPs. DNGR-1$^+$ CDPs exhibit cDC-restricted differentiation potential and do not generate pDCs after adoptive transfer [21••] or *in vitro* culture with Flt3L (BUS and CRS, unpublished observations). DNGR-1$^+$ CDP express CD115, consistent with the recent demonstration that CD115$^+$ CDPs exhibit a strong clonal bias to generate cDCs, whereas pDCs arise predominantly from CD115 negative CDPs [19•]. Thus, cDCs and pDCs appear to have distinct immediate progenitors, which can be distinguished by expression of CD115 [19•] and DNGR-1 [21••]. Some CD115$^+$ CDP, which presumably express DNGR-1 [21••], have combined cDC and pDC potential in clonal assays [19•,30,31], although the interpretation of such experiments might be marred by the reported *in vitro* developmental plasticity of differentiating DCs [35]. Altogether, these data can be integrated into a revised map of DC differentiation that takes into account the fact that cDCs, pDCs and monocytes develop as distinct lineages although the exact developmental intermediates and branching points remain to be clarified and may display considerable plasticity (Figure 1).

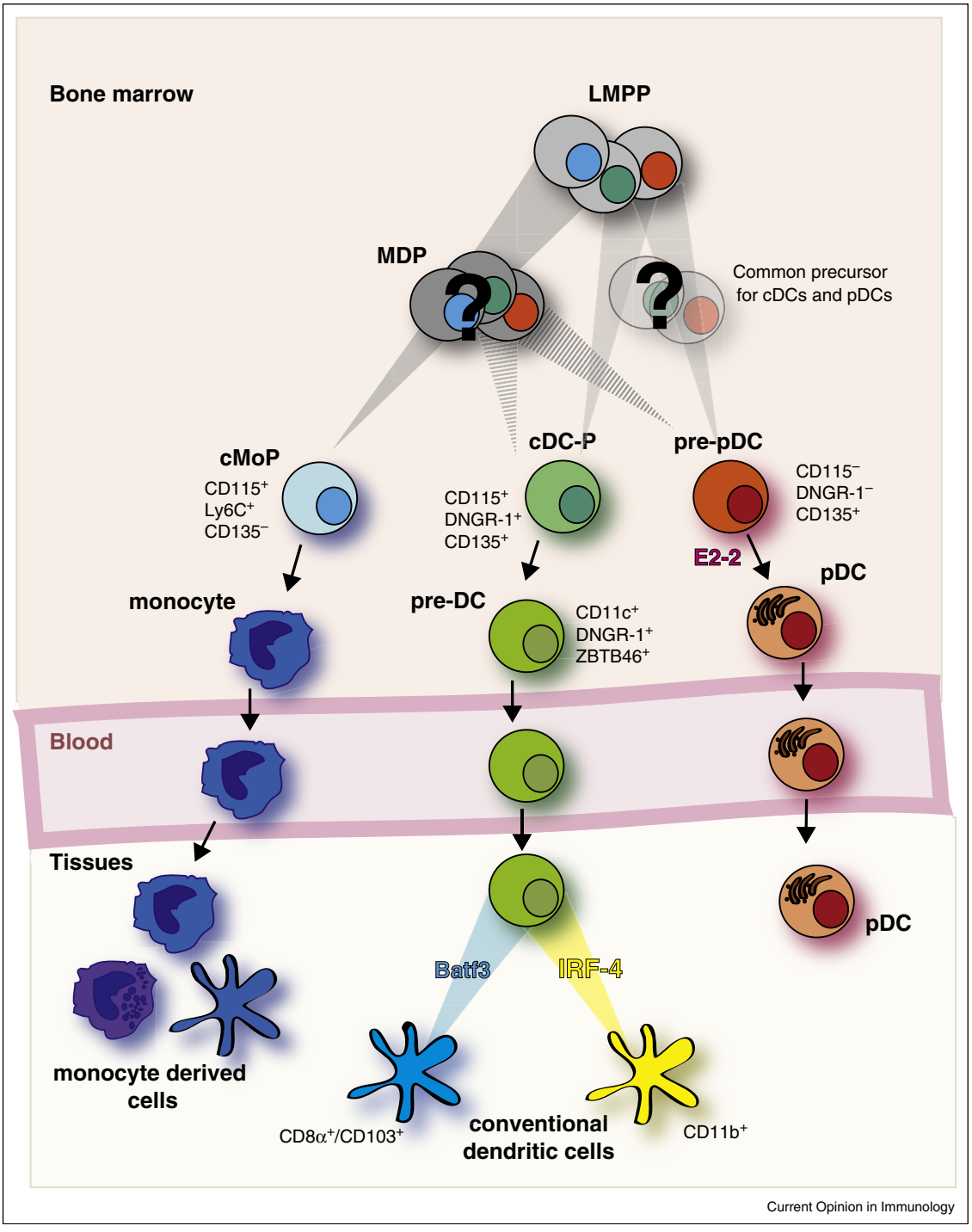
Lineage attribution based on growth factor requirements

Dependence on FLT3L is sometimes used as evidence that a given leukocyte should be considered a member of the DC lineage [36–38]. This is because FLT3L strongly expands pDCs and cDCs *in vivo* [28,39,40] and can be used to generate all functional subsets of DCs *in vitro* [41]. Conversely, mice lacking Flt3L display a severe deficiency in DCs, which is also apparent, although to a lesser extent, in mice lacking its receptor CD135 (Flt3) [42] or treated with CD135 inhibitors [43,44]. GM-CSF, on the other hand, is extensively used to differentiate monocytes into cells resembling DCs *in vitro* [45] but mice lacking GM-CSF or its receptor have normal development of monocyte-derived cells [46•] as well as lymphoid tissue DCs [28,47]. Instead, they exhibit a specific reduction of cDCs in many, but not all, non-lymphoid tissues [46•,48–50]. The GM-CSF dependence of CD103$^+$ cDCs is stronger than that of CD11b$^+$ cDCs [46•] although the extent of reduction relates to the markers used for cell identification [46•,49], possibly because GM-CSF regulates CD103 expression [51]. FLT3L and GM-CSF have also been implicated in the development of human DCs [38,52–55].

Although FLT3L deficiency impacts DC numbers, the cells that do develop in its absence are functional [42]. Transfer of DCs into a FLT3L-deficient environment reduces their homeostatic proliferation [28] suggesting that FLT3L controls peripheral expansion of DCs rather than development. Consistent with that notion, CD135 deficiency has little effect on the number of MDPs in bone marrow and preDCs in spleen [28]. By contrast, preDC frequencies are reduced in non-lymphoid organs of FLT3L deficient mice [36] and CDP numbers also appear affected, although the reported reduction ranges from two-fold [50] to near complete absence [22•] and is further amplified in the absence of GM-CSF [50]. These results are difficult to interpret as FLT3L-deficient mice exhibit abnormalities in various other hematopoietic lineages, including B, T and NK cells [42]. Thus, the exact role of FLT3L in DC development will benefit from the identification of additional receptors for the cytokine and improved genetic tools, such as floxed FLT3 alleles.

Despite being incomplete, FLT3L dependence can still be a useful surrogate for CDP origin. However, a cautionary note is warranted. Even though steady state monocyte development in mice appears FLT3L-independent [42], FLT3L might influence monocyte development into cells that resemble DCs. Indeed, addition of FLT3L to human monocytes cultured in GM-CSF and IL-4 increases the yield of DC-like cells with potent T cell stimulatory capacity [56]. Murine monocytes cultured with FLT3L alone do not become superior stimulators of a mixed lymphocyte reaction [57] but the possibility remains that FLT3L might promote monocyte differentiation into DC-like cells during inflammation *in vivo*, which to our knowledge has not been sufficiently addressed in FLT3L or CD135 deficient animals. Additionally, Langerhans cells (LC), which arise from embryonic progenitors [58,59] and are therefore ontogenetically distinct from DCs, upregulate CD135 expression upon migration to lymph nodes [60•]. Thus, despite their separate ontogeny, FLT3L could help monocytes and LCs assume phenotypic and functional properties generally associated with DCs.

Figure 1



Understanding DC development. Recent data suggest that cDCs, pDCs and monocytes arise via distinct developmental pathways and cellular intermediates. The exact branching points are unclear but lineage imprinting may occur as early as LMPPs, potentially involving epigenetic modifications, indicated by coloured nuclei. The existence of MDP or a common precursor for cDCs and pDCs is debatable. However, cDC-restricted progenitors ('cDC-P') can be distinguished from pDC specific progenitors (pre-pDCs) by expression of CD115 and DNGR-1. cDC-Ps further differentiate into pre-DCs, which acquire CD11c and Zbtb46 expression and travel through the blood to peripheral organs. Their differentiation into $CD8\alpha^+/CD103^+$ and $CD11b^+$ cDCs in target tissues is controlled by the transcription factors Batf3 and IRF-4, respectively. pDC differentiation from pre-pDC is controlled by E2-2 and occurs in the bone marrow. Mature pDCs then enter the blood to seed peripheral organs. Monocytes also develop in the bone marrow and arise from common monocyte progenitors (cMoP), distinguished from cDC-P and pre-pDC by lack of DNGR-1 and CD135 expression and by expression of Ly6C. Monocytes travel via the blood to peripheral organs where they can further change their phenotype and function depending on environmental cues, such as inflammation (monocyte-derived cells).

Lineage attribution based on transcription factors

Demonstrating that the development of a given DC subset requires specific transcription factors has been a powerful way to establish the existence of functionally distinct DC subtypes. We can, for example, distinguish pDCs from cDCs based on the finding that the development of the former but not the latter is dependent on E2-2 [61]. Among cDCs we can further discriminate two main subtypes: CD8α$^+$ cDCs in lymphoid organs and their CD103$^+$ counterparts in non-lymphoid tissues, which depend on IRF8, Id2 and Batf3 [49,62–65], from CD11b$^+$ cDCs, which depend on RbpJ and IRF4 [12••,66,67,68,69••,70••]. Notably, the same transcription factors have been implicated in the development of the likely-equivalent DC subsets in humans [61,71,72], indicating that developmental programs are conserved across species. The transcription factor Zbtb46 (zDC, Btbd4) was identified for its prominent expression in mouse preDCs and differentiated cDCs [37,73•] but is absent from pDCs or their precursors, as well as macrophages and resting monocytes, making it a likely candidate regulator of cDC development [37,73•]. However, Zbtb46 turns out to be dispensable for mouse cDC development [37,73•] even though it might influence DC subset composition [74•]. As Zbtb46 expression is also found in human DCs [75,76], it can nevertheless be a useful marker to identify DCs across species. But its use as lineage defining marker requires caution as Zbtb46 is downregulated after DC stimulation, induced in activated monocytes and expressed in non-immune cells [37,73•]. Interestingly, rather than controlling lineage decisions, Zbtb46 appears to function to reinforce a DC specific transcriptional program [73•] and suppress DC activation [74•]. Notably, mouse monocytes cultured in GM-CSF ± IL-4, uniformly upregulate Zbtb46 [73•]. It will therefore be important to determine whether Zbtb46 controls DC-associated functional attributes of monocyte-derived cells, such as antigen presentation [74•].

Transcriptomic approaches to establish lineage affiliations

Comparative gene expression analyses have identified gene signatures specific to DCs and macrophages and thus can clarify relationships among mononuclear phagocytes [23••,60•,77]. Importantly, transcriptome profiling has helped demonstrate the existence of the same two broad subsets of cDCs across lymphoid and non-lymphoid tissues of both mice and humans [26,38,63,69••,77–80], as well as other species, such as chicken, sheep and pig [81–83]. As such, it is a powerful approach to defining cells, when experimental manipulation is not straightforward or even possible.

It is important to bear in mind that conclusions from global gene expression analysis crucially depend on the homogeneity of the analysed populations and the bioinformatics criteria utilized. For example, *Clec9a* has not been associated with any DC signature [60•] but instead appears in a gene profile unique to red pulp macrophages [77], which express negligible amounts of *Clec9a* mRNA and no DNGR-1 protein (BUS and CRS, unpublished observations). Future studies might circumvent such limitations through the profiling of single cells [84]. More importantly, gene expression profiles might not always be indicative of cell ontogeny. DCs and LCs that have immigrated to lymphoid tissues exhibit striking similarities, independent of tissue of origin [60•]. Therefore, certain transcriptional programs appear regulated by environmental cues rather than cell ontogeny, raising the interesting question of whether these programs reflect functional convergence among phagocytes of distinct hematopoietic origin.

Lineage tracing

Answering the above question requires the development of models allowing for *in vivo* tracing of phagocytes with different origins [63]. By crossing mice expressing Cre recombinase under the control of the *Clec9a* locus to Rosa26-STOP^{flox}-yellow fluorescent protein (YFP) reporter mice, we have recently generated a genetic model with which to fate map the progeny of DNGR-1$^+$ CDP and preDC [21••]. Although *Clec9a*-Cre reporter mice suffer from limitations, as DNGR-1 is also expressed on CD8α$^+$/CD103$^+$ cDCs and to a lower extent on pDCs, we were able to demonstrate YFP expression in DCs but not monocytes or macrophages even after intestinal inflammation and *Listeria monocytogenes* infection [21••]. Importantly, using *Clec9a*-Cre reporter mice, we identified CDP-derived cells within CD64$^+$ cell populations previously thought to represent monocytes/macrophages [21••,85,86]. CD64$^+$ CDP-derived cells are especially frequent in kidneys, where they resemble yolk sac-derived F4/80^{hi} tissue-resident MØs, appear to lack Zbtb46 expression [73•] and where their affiliation as DCs or macrophages has been debated [87]. The presence of a few YFP$^+$ cells in the CD64 component of lung and small intestine indicates the existence of these atypical CDP-derived cells also in other tissues [21••]. Notably, CD64$^+$ kidney DCs stimulated naïve T cells *in vitro*, although less efficiently than CD11b$^+$ cDCs [21••]. Thus, fate mapping of DC precursors reveals previously unappreciated heterogeneity among mononuclear phagocytes, raising the question of why cells of distinct ontogeny but overlapping phenotype exist in the same tissue. More detailed analyses of such atypical CDP-derived cells, for instance by transcriptome profiling, will contribute to elucidating their function and help determine how it is shaped by the local environment.

Conclusion

The classification of DCs based on phenotypic and functional properties that are often shared with other cell types has led to difficulties in cell identification and even debate over the existence of DCs as a discrete leukocyte

lineage. Phenotype-based and function-based definitions are inherently problematic as functional roles and phenotypic markers often change under the influence of environmental cues. While mononuclear phagocyte classification may be considered a semantic issue, it is key to scientific communication. It needs to be robust enough to withstand arguments pertaining to levels of surface marker expression or degree of T cell stimulatory capacity to make it clearly accessible to all researchers inside and outside the field. The identification of distinct developmental precursors underscores that cDCs, pDCs and monocytes constitute separate cell types and enables a move towards cell classification based on ontogeny. Importantly, ontogenetic definitions are independent of functional or phenotypic properties, allowing the investigation of the full spectrum of phagocyte activity in an unbiased manner. This freedom allows us for the first to time to assess to what extent cellular properties are dictated by 'nurture' versus 'nature'. In other words it allows us to determine which functions of tissue mononuclear phagocyte subtypes are determined by ontogeny and which are shaped in the local tissue environment. Understanding DC ontogeny and refining cellular identification is a work in progress that will benefit from improved genetic tools and techniques to analyse single cells.

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