

# Fate mapping of dendritic cells

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Dendritic cells (DCs) are a heterogeneous group of mononuclear phagocytes with versatile roles in immunity. They are classified predominantly based on phenotypic and functional properties, namely their stellate morphology, expression of the integrin CD11c, and major histocompatibility class II molecules, as well as their superior capacity to migrate to secondary lymphoid organs and stimulate naïve T cells. However, these attributes are not exclusive to DCs and often change within inflammatory or infectious environments. This led to debates over cell identification and questioned even the mere existence of DCs as distinct leukocyte lineage. Here, we review experimental approaches taken to fate map DCs and discuss how these have shaped our understanding of DC ontogeny and lineage affiliation. Considering the ontogenetic properties of DCs will help to overcome the inherent shortcomings of purely phenotypic- and function-based approaches to cell definition and will yield a more robust way of DC classification.

**Keywords:** dendritic cell, ontogeny, fate mapping, lineage tracing, mononuclear phagocyte

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## Introduction

Dendritic cells (DCs) were originally identified in mouse spleen for their unique stellate morphology, their ability to adhere to certain glass surfaces and their superior capacity to activate naïve T lymphocytes that distinguished them from macrophages (MØs) (1–3). Mostly for historical reasons, DCs are considered part of the mononuclear phagocyte (MP) system, which groups all highly phagocytic cells derived from monocytes or their precursors based on the premise that tissue MØs arise from monocytes (4–9). This presumed relatedness of DCs, monocytes, and MØs coupled to the lack of reliable ways to distinguish MP subtypes has caused continuous debates over accurate cell-type identification and has led some to question whether DCs in fact constitute an independent cell lineage (6, 7, 10–14). However, today we have conclusive evidence demonstrating that DCs, monocytes, and MØs have distinct cellular origin and we further distinguish plasmacytoid DCs (pDCs) from two subsets of so-called conventional or classical DCs (cDCs) based on unique developmental requirements (7, 15–19). Nonetheless, DCs remain defined based on phenotypic and functional properties that often overlap with those of monocytes or MØs (19), although some have suggested a shift in paradigm toward a nomenclature that takes cell ontogeny into account (6, 7, 10).

Dendritic cells are generally identified by their high expression of major histocompatibility complex class II molecules (MHCII) and of the integrin CD11c, as well as their superior capacity to migrate from non-lymphoid to lymphoid organs and stimulate naïve T cells (3, 20–22). However, these characteristics are not absolute and can change in situations of inflammation or infection, thus complicating cell identification (6, 7, 23, 24). For instance, CD11c, considered the hallmark surface marker of DCs, is also found on B, T, and NK cells as well as some monocytes, MØs, and eosinophils (25–32). Dendritic protrusions have also been observed in some MØs and T cells (33–35). Further, surface markers, such as F4/80, CD14, or CD64 (Fc-gamma receptor 1), generally associated with monocytes or MØs can be found on DCs (36–38). One might argue that the most defining feature of DCs is their ability to activate T cells, however such definition discounts the fact that DCs potentially

regulate innate immune responses independent of their ability to migrate to lymphoid organs or stimulate T cells (39–44). Conversely, non-DCs can carry antigen to lymph nodes and activate naïve T cells in some instances (45–47).

Therefore, morphological and functional properties, as well as the expression of surface markers are insufficient to clearly distinguish DCs from monocytes and MØs, raising the necessity to find a more robust way of cell identification. Recent studies in mouse and human indicate that DCs, MØs, and monocytes have unique ontogenetic properties and thus can be considered distinct cell lineages (36, 48–54). Here, we review approaches that have been employed to track and define the progeny of DC precursors *in vivo* and discuss how such “fate mapping” approaches have improved our understanding of DC heterogeneity and ontogeny. These studies lay the foundation for moving toward cell ontogeny as a major lineage-determining criterion, which will allow for a more reliable and precise classification of DCs and DC subsets.

## DC Development

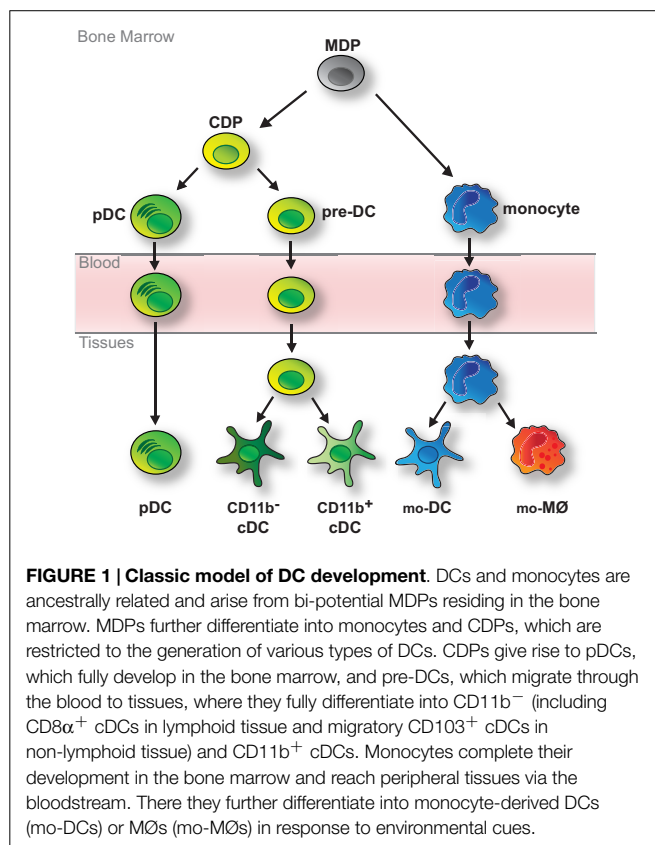
Dendritic cells are short-lived and their maintenance relies on constant replenishment from bone marrow progenitors that originate from hematopoietic stem cells (HSCs) (19, 55). In the classic model of DC development monocytes and DCs arise from bi-potent progenitors, so-called MØ and DC progenitors (MDPs) (Figure 1) (56). MDPs further give rise to common DC progenitors (CDPs) restricted to the generation of pDCs and cDCs (Figure 1) (57, 58). pDCs terminally differentiate in the bone

marrow, thus exit the bone marrow as fully developed cells and reach peripheral organs via the blood stream (Figure 1) (15, 59). In contrast, cDCs arise from another developmental intermediate termed pre-DC, which exits the bone marrow and migrates through the blood to seed lymphoid and non-lymphoid tissues (60, 61). There, pre-DCs terminally differentiate into cDCs, including the main CD11b<sup>-</sup> and CD11b<sup>+</sup> subtypes (Figure 1) (60–63). In lymphoid tissues these are CD8α<sup>+</sup>CD11b<sup>-</sup> and CD11b<sup>+</sup> resident cDCs, whereas in non-lymphoid tissues they comprise CD103<sup>+</sup>CD11b<sup>-</sup> and CD11b<sup>+</sup> migratory cDCs (3, 60–63). Like pDCs, monocytes complete their development in the bone marrow but in tissues they differentiate into cells with DC- or MØ-like features (Figure 1) (23, 24, 64, 65). This plasticity is remarkably prominent in inflammatory or infectious environments, when monocyte-derived cells with qualities of DCs have been referred to as TNF-α/iNOS-producing DCs (Tip-DCs), monocyte-derived DCs (mo-DCs), and/or inflammatory DCs (23, 24, 64, 65).

Although most of our knowledge concerning DC development is derived from mouse studies, developmental parallels have been observed in other species (66–73). Especially the identification of putative equivalent DC progenitor populations in human holds promise for future research (72, 73). Yet, some uncertainties remain. Common lymphoid progenitors (CLPs) can give rise to DC descendants upon adoptive transfer (74), although it is now thought that DCs originate predominantly from myeloid progenitors (75, 76). Nonetheless, some pDCs, but not cDCs, show evidence of VDJ gene rearrangements, potentially indicating lymphoid lineage heritage (15, 59, 77). However, it remains unclear whether evidence of *Rag* gene expression history necessarily means that pDCs have dual lymphoid and myeloid origin. Contrary to the dogma that monocytes and DCs share a common immediate ancestor, recent data suggest that lineage divergence of HSC-derived myeloid cells occurs much earlier than previously predicted and that monocytes and DCs might arise independent of a bi-potent developmental intermediate (49, 78, 79). Elucidating such unresolved aspects pertaining to DC ontogeny may solve uncertainties in determining lineage affiliation, which, in turn, will aid to further decipher the unique functions of DCs in immunity.

## Fate Mapping

Understanding cell development requires models with which the relationship of a precursor cell and its progeny can be defined *in vivo*. Such “fate mapping” can be achieved in various ways and relies on the selective labeling of the cell(s) of interest so that consequently the development of the marked cell can be followed in its natural environment (80). Tracing progenitors *in vivo* also offers the possibility to determine the fate of populations when lineage affiliation is most heavily debated, namely following experimental manipulation to generate conditions of inflammation or infection. While most fate mapping strategies follow the progeny of bulk cell populations, recently developed techniques have enabled the tracing of single cells, thus providing valuable information regarding their developmental potential at the clonal level (80, 81). In all fate mapping experiments, it is important to consider that their



interpretation is dependent on the use of select, faithful and stable markers (82).

### Precursor Transfers

The transfer of purified and pre-marked precursor cells into congenic recipients is the most accessible form of fate mapping as a variety of labeling options can be used to distinguish between donor and host cells (Figure 2A) (80). As a result, precursor transfers are commonly used to study cell development and lineage relationships and remain a standard protocol for defining the stemness of progenitor cells (80). Such experiments rely on the ability to purify sufficient precursors that, after cell isolation, retain the capacity to home to the appropriate anatomical niche and expand sufficiently into detectable progeny. To circumvent such limitations transfer studies are often combined with protocols to induce leukopenia, such as irradiation, in order to increase the niche available for cell engraftment (Figure 2A) (80). However, these manipulations can alter developmental signals, which, in turn, might impact on the interpretation of results (18, 54, 83). To best mimic the endogenous cellular environment, progenitors have been returned directly to their organs of origin, for instance by intra-bone injection (84).

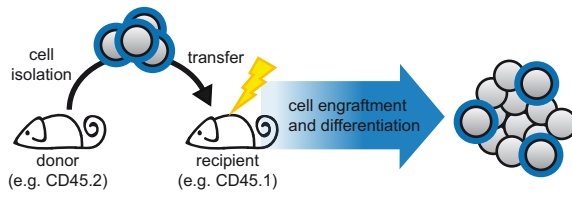
The DC progenitors MDP, CDP, and pre-DC were in part defined by assessing their developmental potential after adoptive transfer into mice (56–58, 60, 61, 84–86). In such experiments, MDPs give rise to DCs and monocytes, whereas CDPs and pre-DCs are restricted to the generation of DCs but do not generate monocytes or other leukocyte lineages (56–58, 61, 84–86). In combination with experiments assessing the differentiation potential of single progenitors *in vitro* (56–58), these studies have significantly shaped our view of DC development (Figure 1). Surprisingly, the existence of MDP as a bi-potential intermediate for DCs and monocytes has recently been questioned when single CX<sub>3</sub>CR1<sup>+</sup> MDPs were unable to generate both DCs and monocytes upon differentiation *in vitro* (78). The authors further found that adoptively transferred CX<sub>3</sub>CR1<sup>+</sup> MDPs, not only gave rise to DCs and monocytes but also neutrophils (78). However, such multi-potency of MDPs was not observed in earlier studies (52, 56, 61, 85, 86) and is not evident in genetic CX<sub>3</sub>CR1 fate mapping experiments (50). It is possible that these discrepancies may be explained by experimental variation such as differences in cell isolation, the timing of analysis or variances in the niche available for cell engraftment following irradiation (18, 54, 83). In light of these results it is noteworthy, however, that upon adoptive transfer MDPs exhibit pDC potential only in some studies (52, 86) but not others (56, 85), whereas the presumed downstream CDPs produce both pDCs and cDCs (57, 58). Taken together these experiments raise some doubt about the existence of a MDP as a key developmental intermediate for monocytes, cDCs, and pDCs. However, resolving this matter will require the use of better models to trace single cells *in vivo* as experiments relying on the isolation and analysis of bulk progenitor populations are inherently prone to disparities in gating strategy or cell purity.

In DC ontogeny, these issues are augmented because MDP and CDP exhibit substantial phenotypic overlap: both lack lineage-defining markers, are characterized by expression of CX<sub>3</sub>CR1, CD115 (M-CSFR, Csf1R) as well as CD135 (FMS-like tyrosine

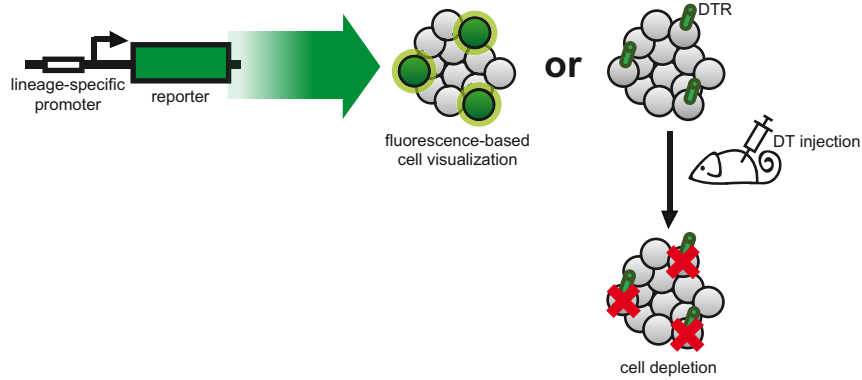
kinase 3, FLT3) and, until recently, CDP could only be distinguished from MDP by lower expression of the receptor tyrosine kinase CD117 (c-kit) (56–58, 61, 86). We have recently found that the C-type lectin receptor DNNGR-1 (Clec9a) marks cells resembling CDPs (36). Surprisingly, upon adoptive transfer, DNNGR-1<sup>+</sup>CD115<sup>+</sup> progenitors exhibit cDC-restricted differentiation potential and do not generate pDCs (36), suggesting that DNNGR-1 marks cDC-restricted progenitors. These data are in line with a recent study demonstrating a strong bias for CD115<sup>+</sup> CDPs to generate cDCs, whereas pDCs arise predominantly from CD115 negative cells (79). Therefore, cDCs and pDCs appear to have distinct developmental intermediates that can be distinguished by expression of CD115 (79) and DNNGR-1 (36). Since CD115<sup>+</sup> CDPs presumably express DNNGR-1 (36), it is unclear why some CD115<sup>+</sup> CDPs show combined cDC and pDC potential in clonal assays (57, 58, 79). It is possible that antibody-mediated triggering of DNNGR-1 or growth factor receptors, such as CD115, during cell isolation skews DC differentiation toward a particular DC sub-lineage in an unforeseeable manner. The developmental potential of progenitors may also be influenced by the specific culture conditions used (78) or DCs could exhibit a degree of developmental plasticity (87). Nonetheless, the existence of a putative intermediate monocyte-restricted progenitor downstream of MDP (common monocyte progenitor, cMoP) (52) alongside the aforementioned pDC- and cDC-restricted progenitors supports a model in which monocytes, cDCs, and pDCs develop independently. The genuine point of lineage divergence, however, remains to be determined.

Questions regarding the lineage affiliation of DCs have been muddled significantly by the developmental plasticity of monocytes (6, 24). The phenotypic transformation of monocytes into DC-like cells is most prominent in inflamed environments (8, 19, 23, 24). It can also be mimicked *in vitro* by culturing monocytes in the presence of GM-CSF (granulocyte-macrophage colony-stimulating factor) ± IL-4 (Interleukin-4) (88, 89). However, *in vivo* the inflammation-induced differentiation of monocytes into cells with attributes of DCs appears GM-CSF-independent (90), highlighting that the developmental requirements underlying this phenotypic conversion *in vitro* might differ from those involved *in vivo*. In the absence of experimentally induced infection or inflammation, adoptively transferred monocytes readily acquire CD11c and MHCII expression as well as functional features of DCs in non-lymphoid tissues (91–95). This phenotypic conversion is also observed after adoptive transfer into unirradiated hosts, which most closely mimics steady-state conditions (63). In contrast, transferred monocytes do not generate DCs in lymphoid organs, even if the niche for engraftment is opened by depletion of CD11c<sup>+</sup> cells (84). Importantly, in non-lymphoid tissues monocytes exclusively generate CD11b<sup>+</sup>, but not CD103<sup>+</sup>CD11b<sup>-</sup> cells, which is in contrast to CDPs and pre-DCs that generate CD11b<sup>+</sup> as well as CD103<sup>+</sup>CD11b<sup>-</sup> cDCs (63, 91–95). Therefore, CD11c<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>+</sup> cells in non-lymphoid tissues appear to constitute a population of mixed cellular origin that can arise from monocytic progenitors as well as pre-DCs. Adoptive transfer experiments do not allow to determine the relative contribution of each progenitor to this population,

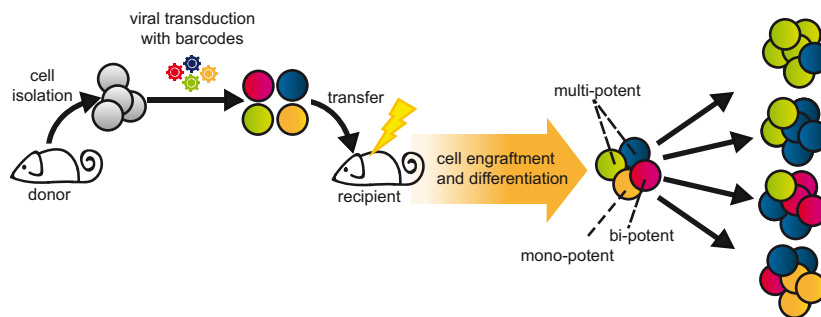
**A Precursor transfer**



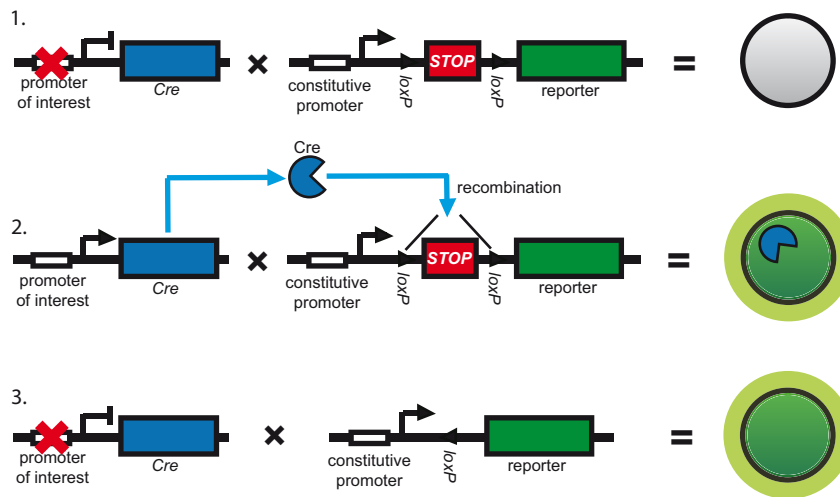
**B Reporter genes**



**C Cellular barcoding**



**D Genetic lineage tracing**



**FIGURE 2 | Strategies to fate map DCs.**

(Continued)

**FIGURE 2 | Continued**

**(A)** Progenitors are adoptively transferred to assess their differentiation in the physiological context. Graft-derived cells are distinguished from host cells based on pre-defined labels, for instance congenic markers. This method is often combined with strategies to increase the niche available for cell engraftment, such as irradiation. **(B)** In transgenic approaches, lineage-restricted promoters can be used to drive a reporter gene. Target cell populations can be visualized by the expression of fluorescent proteins or can be depleted. In the latter case, cell-restricted expression of DTR allows for conditional cell ablation following DT injection. **(C)** Progenitors are transduced *in vitro* with semi-random DNA sequences (barcodes) by retro- or lentiviral vectors and subsequently

transferred into irradiated congenic recipients. After differentiation, cell progeny are analyzed for their barcode repertoire using deep sequencing or microarray. The representation of a given barcode in multiple cell populations indicates multi-potency of the transferred cell. **(D)** Expression of Cre recombinase is driven by a lineage or cell-specific promoter. Additionally, a reporter gene, usually a fluorescent protein, is placed under control of a constitutive promoter. Expression of the reporter is blocked by inserting a *loxP*-flanked *STOP* cassette (1). Cre expression leads to excision of the *STOP* cassette resulting in expression of the reporter gene (2). Since the promoter-driving reporter gene expression is constitutively active, the target cell is irreversibly labeled irrespective of continuous Cre expression (3).

although surrogate markers such as CD64 or Mar-1 can serve to distinguish monocyte-derived cells from bona fide pre-DC-derived cDCs (46, 93, 94).

Notably, in irradiated hosts transferred monocytes can also generate CD11c<sup>+</sup>MHCII<sup>+</sup> cells of the epidermis, which resemble Langerhans cells (LCs) (96–99). LCs exhibit many phenotypic and functional features of DCs, such as the capacity to migrate to lymphoid organs and stimulate naïve T cells, and have long been considered a prototypical DC population (96–99). However, we now realize that the majority of LCs is established before birth and maintained under steady-state conditions by self-renewal from local progenitors (96, 97, 99–102). These properties thus ontogenetically separate LCs from bone marrow-derived DCs or monocyte-derived cells. Moreover, monocytes may not necessarily adopt features of DCs or MØs upon entry into tissues, as a recent study indicates that monocyte can also exist in tissues without further differentiation (45). When considering this immense plasticity it will be crucial to elucidate the environmental cues that shape the diverse fates of monocytes to further dissect the full functional spectrum of monocytes and monocyte-derived cells.

### Lineage Restricted Reporters

When the availability of isolatable progenitor cells is limiting and when populations are ontogenetically heterogeneous or might be influenced by alterations in their surroundings, determining lineage affiliation requires models to trace cells directly in their natural environment. One way to achieve this is by engineering models in which lineage-restricted promoters or genetic elements drive the expression of reporter genes (**Figure 2B**) (80, 82, 103). It is important to bear in mind that such experiments assume that the expression of the selected marker is restricted to the cell lineage in question and therefore, the choice of stable and specific markers is essential (80, 82, 103). Additionally, the genetic elements used to drive expression of the reporter must faithfully mimic endogenous gene expression (80, 82, 103).

Genetic elements of the *Itgax* gene, which encodes CD11c, have extensively been used to generate reporters to study DCs (82, 103). As such, transgenic mice in which the CD11c promoter drives the expression of fluorescent proteins (**Figure 2B**) have been key to visualizing the distribution and cellular interactions of DCs in a variety of tissues, including lymphoid organs, heart, lung, and skin (103–107). But fate mapping can also be achieved by cell deletion. Transgenic expression of primate diphtheria toxin receptor (DTR) renders murine cells susceptible to diphtheria toxin (DT)-induced cell death and, thus, enables inducible target

cell depletion (**Figure 2B**) (82, 108). In this sense mice in which DTR expression is controlled by the elements of the CD11c promoter have been widely used to characterize the *in vivo* functions of DCs (28, 109–112). In part through analyzing such reporter mice, however, it has become evident that CD11c expression is not entirely restricted to DCs. It is also expressed on alveolar MØs, Ly6C<sup>low</sup> as well as activated monocytes, plasmablasts, NK cells, and some T cells (25–29, 113). In addition, CD11c-driven fate reporter expression varies depending on the specific promoter elements used for transgenesis. CD11c.DTR mice, which were generated by conventional transgenesis using a 5.5-kb promoter element of the *Itgax* gene (109, 114), efficiently deplete most CD11c-expressing cDCs, LCs, alveolar, splenic marginal zone, and metallophilic MØs, as well as plasmablasts and T cells (27, 109, 115). However, DT-induced cell depletion in these mice is incomplete and spares certain cell types that transcribe their endogenous *Itgax* allele, including pDCs and NK cells (82, 115). Additionally, prolonged cell depletion using CD11c.DTR mice requires the use of bone marrow chimeras, possibly because of aberrant DTR expression on non-immune cells (82, 108, 112). Notably, this is not the case in CD11c.DOG and CD11c.LuciDTR mice, which were generated using bacterial artificial chromosome (BAC) transgenesis to place DTR under control of the extended regulatory region of the *Itgax* gene and in which DTR expression seems to more faithfully represent endogenous CD11c expression (28, 112, 115, 116). In all models, the occurrence of systemic neutrophilia and monocytosis following CD11c<sup>+</sup> cell depletion (28, 115, 117) adds another layer of complexity to deciphering the cellular function and lineage affiliation of DCs.

The realization that CD11c is not restricted to DCs in all instances nurtured the search for more specific lineage-defining markers. Two groups simultaneously identified the transcription factor *Zbtb46* (zDC, *Btbd4*) as ideal candidate to distinguish cDCs, as it is expressed in pre-DCs and cDCs but not in pDCs or their precursors (37, 38). Consistently, CD8α<sup>+</sup> and CD11b<sup>+</sup> cDCs in lymphoid organs as well as CD103<sup>+</sup> cDCs in non-lymphoid organs uniformly express *Zbtb46* as assessed in *Zbtb46*-GFP (37) and *Zbtb46*-DTR (38) reporter mice generated by site-directed mutagenesis. In contrast, CD11c<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>+</sup> cells in non-lymphoid organs, including lung, small intestine, and kidney, exhibit partial *Zbtb46* expression (37, 38) indicating that they represent a heterogeneous population. This is consistent with reports demonstrating that these cells are of mixed monocyte and pre-DC origin (63, 91, 92, 95). Subsequently, *Zbtb46* reporter mice have been used to help establish lineage relationships in a variety of tissues including



heart, pancreas, tumors, and thymus (118–121). The fact that *Zbtb46* expression is also found in human DCs suggests that it may also help to identify DCs across species (48, 122).

Nevertheless, the use of *Zbtb46* as lineage-defining marker requires a note of caution. *Zbtb46* expression is downregulated after DC stimulation and it is found in some non-immune cells (37, 123). Despite its prominent expression in the cDC lineage, *Zbtb46* appears largely dispensable for cDC development (37, 123). Instead, it may reinforce DC-specific transcriptional programs (37) and/or suppress DC activation (123). Interestingly, monocytes activated in the presence of GM-CSF ± IL-4 uniformly induce *Zbtb46* expression, whereas monocyte-derived Tip-DCs that are generated following infection with *Listeria monocytogenes* do not (37). This raises the possibility that *Zbtb46* may control DC-like features of monocyte-derived cells in some inflammatory situations and it will be interesting to determine if *Zbtb46* controls transcriptional programs in monocytes. These data also highlight that despite its selective expression on cDC progenitors and their descendants, *Zbtb46* is not necessarily an indicator of cell ontogeny.

### Identifying Common Developmental Requirements

Establishing that the development and/or delineation of a cell type depends on a certain transcription or growth factor constitutes a powerful way of fate mapping that has extensively been applied to MPs (42, 51, 63, 124–141). We can now clearly delineate DCs into distinct subpopulations based on the transcriptional programs that govern their development. pDCs are distinguished from two subsets of cDCs by their dependence on E2-2 (67, 142). The differentiation of pre-DCs into CD8 $\alpha$ <sup>+</sup> cDCs in lymphoid organs and CD103<sup>+</sup>CD11b<sup>-</sup> cDCs in non-lymphoid tissues is controlled by a set of transcription factors, including Irf8, Nfil-3, Id2, and Batf3 (124–128). Therefore, CD8 $\alpha$ <sup>+</sup> cDCs and CD103<sup>+</sup> cDCs represent a developmentally related lineage of cDCs (6, 7). Notably, these cells also exhibit a degree of functional relatedness that is, for instance, exemplified by their superior capacity to activate CD8<sup>+</sup> T cells (124, 143–145). In contrast, the development of CD11b<sup>+</sup> cDCs from pre-DCs is controlled by distinct transcription factors, including RelB, RbpJ, PU.1, and Irf4 (42, 129–136). Notably, expression of CD24 separates pre-DCs into cells that preferentially generate either CD8 $\alpha$ <sup>+</sup> or CD11b<sup>+</sup> cDCs in spleen (60) suggesting a stepwise differentiation of pre-DCs into cDCs. It will be interesting to determine whether such heterogeneity of pre-DCs also exists in the bone marrow. Notably, the extent of transcription factor dependence is linked to the genetic background of the particular mouse strain analyzed (146–148), indicating that transcriptional requirements are not always absolute or redundant factors exist (148). Consistently, CD8 $\alpha$ <sup>+</sup> DCs can develop in the absence of Batf3, Id2, and Nfil-3 (149). The local microenvironment may also contribute to shaping the diversity of the DC compartment, as in some tissues, such as the spleen and intestinal system, CD11b<sup>+</sup> cDCs can be divided into ontogenetically and functionally distinct subpopulations (36, 42, 91, 95, 131). Importantly, some of the transcription factors controlling DC differentiation in mice have also been implicated in the development of human DCs (67, 69, 71) and putative equivalent

DC subpopulations exist in rat, chicken, sheep, and pig (150–153), highlighting that DC populations are conserved across species.

While several growth factors have been linked to DC differentiation, the development of all DC subsets is strongly dependent on FLT3 ligand (FLT3L) and downstream signaling events (7, 18, 154). FLT3L administration potently expands pDCs and cDCs in mice and humans (72, 73, 85, 155–157). *In vitro*, FLT3L promotes the differentiation of bone marrow progenitors from mice, humans, and pigs into functional subsets of DCs (66, 158, 159). Mice lacking FLT3L display a severe deficiency in DCs, which is also apparent, although to a lesser extent, in mice lacking its receptor CD135 or mice treated with CD135 inhibitors (63, 137, 160, 161). In contrast, FLT3L appears largely dispensable for monocyte and M $\phi$  development (137) and, therefore, FLT3L dependency is often used to delineate DCs *in vivo* (18, 65, 162). The interpretation of fate mapping using mice deficient in CD135 or its ligand is however complicated by the fact that these animals also exhibit abnormalities in other hematopoietic lineages, including B, T, and NK cells (137, 163) and show evidence of systemic neutrophilia and monocytosis, as has been reported in other DC-deficient models (112, 117).

Despite the prominent expression of CD135 on DC progenitors it remains to be clarified exactly at what stage of cellular differentiation FLT3L impacts on DC development. Consistent with a role for FLT3L early in development, a reduction of bone marrow CDPs in FLT3L deficient animals has been reported but ranges from a mere twofold decrease (164) to near complete absence (78). In contrast, the numbers of MDPs and splenic pre-DCs appear largely unaffected by CD135 deficiency (85). The observation that pre-DC frequencies in non-lymphoid organs of FLT3L-deficient mice are reduced (63) and that transfer of DCs into a FLT3L-deficient environment decreases their homeostatic proliferation (85) indicates a role for FLT3L in the peripheral expansion of DCs rather than their differentiation. This interpretation would equally be consistent with the observation that DCs that develop in the absence of FLT3L are functional (137). In light of this finding it will be interesting to determine, to what extent FLT3L impacts on the development and functional regulation of other MPs. Addition of FLT3L to purified human monocytes cultured with GM-CSF ± IL-4 increases their T cell stimulatory capacity (165), although it is not clear whether this is also the case for murine monocytes. Culture of murine bone marrow with GM-CSF and IL-4 presumably mimics monocyte differentiation under the same conditions (166). When FLT3 signaling is inhibited in such bulk cultures the T cell stimulatory capacity of the output cells is reduced (161). Therefore, these data raise the possibility that FLT3L might influence monocyte differentiation into cells with functional properties of DCs also in the murine system, although a direct causality remains to be demonstrated. Further, comparative gene expression profiling revealed that upon migration to lymph nodes LCs induce CD135 expression (167), indicating that they might be capable of responding to FLT3L. Therefore, it is conceivable that FLT3L may control certain functional aspects generally associated with DCs, such as antigen presentation, in ontogenetically distinct MP subtypes, which will be interesting to formally address in the context of FLT3L or CD135 deficiency.

Dendritic cell progenitors also express CD115, the receptor for M $\phi$  colony-stimulating factor (M-CSF) (56–58, 61, 86). However, compared to the dominant role of FLT3L in DC differentiation, M-CSF-deficiency only mildly impacts on DC development (168). M-CSF deficient osteopetrotic (op/op) mice exhibit a two- to threefold reduction in splenic cDCs and pDCs, respectively, but the remaining DCs are capable of stimulating a mixed lymphocyte reaction and induce costimulatory molecules upon activation, thus appear functional (168). In contrast, M-CSF is strongly required for monocyte and M $\phi$  development (141, 169). Therefore, the observation that mice lacking CD115 exhibit reduced frequencies of CD11c<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>+</sup> cells in non-lymphoid organs (63, 91) likely reflects the ontogenetic heterogeneity of this population (63, 91–95). Consistently, M-CSF is also required for the generation of monocyte-derived cells with features of DCs during inflammation (90). Nonetheless, M-CSF may play a role in DC development. It can promote DC differentiation *in vitro* and *in vivo* even in the absence of FLT3L, although DCs generated by M-CSF alone phenotypically and functionally differ from those induced by FLT3L (170). M-CSF-induced DC poesis is also more efficient in FLT3L-sufficient conditions (170). *In vivo*, antibody-mediated blockade of M-CSF in pregnant mice reduces pre-DC extravasation, translating into a reduction of CD11b<sup>+</sup> DCs in the pregnant uterus (171). Whether M-CSF affects pre-DC migration also in other tissues and whether it acts in a cell intrinsic manner or by promoting the production of chemotactic factors by other cells remains to be determined (171).

In purified monocytes, GM-CSF induces phenotypic and functional attributes of DCs (88, 89, 172). Similarly, purified CD115<sup>+</sup> MDPs respond to GM-CSF by differentiating into CD11c<sup>+</sup>MHCII<sup>+</sup> DCs (85) and GM-CSF deficiency leads to a slight reduction of bone marrow MDPs and CDPs (164). However, GM-CSF is dispensable for the differentiation of lymphoid tissue DCs (85, 173) and, therefore, it seemed likely that GM-CSF would selectively regulate the differentiation of monocytes into cells resembling DCs (23). This speculation also led to the hypothesis that monocytes cultured in the presence of GM-CSF represent the counterpart of mo-DCs generated under conditions of inflammation/infection *in vivo* (23). Surprisingly, GM-CSF does not appear to control monocyte differentiation *in vivo* (90) and thus, GM-CSF elicited monocyte-derived cells are unlikely to be fully equivalent to inflammatory monocyte-derived cells. Rather, GM-CSF influences the homeostasis of cDCs in a variety, but not all, non-lymphoid tissues, most likely by promoting cell survival (90). Importantly, GM-CSF deficiency leads to a greater reduction of CD103<sup>+</sup> cDCs than of CD11b<sup>+</sup> cDCs (90). However, the extent of cDC reduction in the absence of GM-CSF apparently relates to the markers used for cell identification (90, 147, 164). This is most likely because GM-CSF regulates certain phenotypic as well as functional features of DCs, such as CD103 expression (174) or their ability to cross-present antigen (90, 174, 175). Therefore, the above-mentioned growth factors not only influence lineage decisions but also impact on the functional regulation of DCs, monocytes, and M $\phi$ s. Elucidating the exact roles of FLT3L, GM-CSF, and M-CSF in each cell type will help to decipher the functional heterogeneity of MDPs.

## Cellular Barcoding

The biggest challenge for fate mapping is to trace the developmental plasticity of individual cells. This can now be achieved using “cellular barcoding,” in which progenitors are tagged *in vitro* with semi-random, non-coding DNA sequences by transduction using retro- or lentiviral vectors (Figure 2C) (81). Therefore, the barcodes are heritable and by choosing conditions of low transduction efficiency one can ensure that each cell receives only a single barcode. Subsequently, barcode-labeled progenitors are adoptively transferred in numbers low enough to minimize the chance that two identically barcoded cells are transferred into the same recipient (Figure 2C). After differentiation *in vivo*, cell progeny are analyzed for their barcode repertoire using deep sequencing or custom microarray. Since each barcode represents an individual progenitor, the presence of the same barcode in more than one cell type indicates that they were generated from a single precursor (multi-potent or bi-potent, Figure 2C). On the other hand, if a barcode is only found in one cell type, the progenitor generated only a single cell lineage (mono-potent, Figure 2C) (81).

During maturation, HSCs are thought to progressively lose their self-renewal ability and become increasingly limited in their differentiation potential, ultimately giving rise to lineage-restricted progenitors (55, 176). Lymphoid primed multi-potent progenitors (LMPPs) are developmental intermediates downstream of HSCs that can give rise to various, but not all, cell lineages and are thus considered multi-potent (55, 176). Surprisingly, in barcoding experiments only a minority (3%) of single LMPPs exhibits true multi-potency, defined as the ability to generate all of the following cell lineages: B cells, DCs, and myeloid cells (monocytes and neutrophils) (49). Rather, single LMPPs differ drastically in terms of their cellular output: 10% of the progenitors contribute primarily to B cells, 10% primarily to myeloid cells but about 50% of transferred LMPPs produce predominantly DCs (49). The remaining fraction of progenitors exhibits bi-potentiality to generate combinations of the examined cell lineages (49). Therefore, LMPPs are multi-potent when analyzed as a population, however single cells exhibit unexpected lineage bias that is imprinted early in development. Why the majority of LMPPs is DC-committed (49), even though DCs constitute a minority lineage compared to B cells, remains to be clarified, although it is possible that some progenitors proliferate better than others or have certain competitive advantages. A major lineage divergence toward DCs seems to occur before or at the LMPP stage, as most HSCs analyzed by the same method are multi-potent, although even HSCs exhibit a degree of lineage bias (49, 177). Since CDPs might arise directly from LMPPs without additional developmental intermediates (79), these data infer that DCs diverge as a developmental lineage distinct from other myeloid cells early on (49).

This, again, questions the existence of a bi-potential MDP as central intermediate in the development of DCs and monocytes. Yet, it is noteworthy that even though DC-biased LMPPs are fivefold more frequent than bi-potent myeloid/DC LMPPs, mono-potent and bi-potent progenitors contribute equally to the final DC pool (49). Therefore, bi-potent progenitors seem to play a significant part in generating DCs, potentially because they have a

proliferative advantage. Resolving these issues will require further refinement of the technique at hand. The differentiation potential of progenitors may be influenced by cell isolation, processing or *in vitro* manipulation (80) and virus-mediated transformation might skew cell fate in an unforeseeable manner, as evidenced by the fact that barcoded LMPPs cannot generate T cells (49, 81). This also means that barcoding does not yet uncover the full potential of single progenitors. The early lineage bias of HSCs and LMPPs suggests that cell development may follow a model of graded commitment rather than proceeding in a truly stepwise manner (178). It will be interesting to determine, to what extent this process is regulated by epigenetic modification and how inflammatory processes might impact on lineage divergence. Future studies will benefit from the development of models allowing for *in vivo* barcoding of single cells but the labor-intensive quantification and analysis of barcoding experiments makes it difficult to follow populations in real time.

### Genetic Lineage Tracing

Dynamic mapping of populations of distinct origin *in vivo* can be achieved using genetic lineage tracing based on *Cre-loxP* technology (Figure 2D) (80, 179). It relies on inducible reporter genes that are placed under the control of constitutively active promoters, such as the *Rosa26* locus. The reporter is most commonly a fluorescent protein that is preceded by a *loxP*-flanked *STOP* cassette and, therefore, its expression is induced only after Cre recombinase (Cre) mediated excision of the stop codon (Figure 2D). Since this form of labeling is genetic it is also heritable, meaning that any cell expressing Cre will pass on the label to all progeny, irrespective of continuous recombinase expression (Figure 2D). Since the promoter driving the reporter gene is constitutively active, labeling is irreversible and not affected by fluctuations in gene expression (Figure 2D) (80).

By crossing mice expressing Cre under the control of the *Clec9a* locus to *Rosa26-STOP-flox-enhanced-yellow* fluorescent protein (YFP) reporter mice (180), we have recently generated the first genetic model to trace the progeny of DNGR-1<sup>+</sup> CDPs and pre-DCs (36). In these mice, YFP expression is restricted to DCs but is not found in monocytes or MØs even in inflammatory conditions, as tested after intestinal inflammation or infection with *L. monocytogenes* (36). Nonetheless, certain limitations need to be taken into account. DNGR-1 is also expressed on CD8α<sup>+</sup>/CD103<sup>+</sup> cDCs and to a lower extent on pDCs (36, 71, 181, 182) and, therefore, in these populations labeling is not a strict indicator of cell ontogeny. Further, labeling of CDP and pre-DC progeny in mice heterozygous for Cre is incomplete, possibly due to a delay in Cre protein synthesis and DNA recombination in rapidly cycling progenitors (36). Consistently, penetrance of the YFP label is increased in mice homozygous for Cre (36). The efficiency of lineage tracing experiments in such cases or when Cre expression is low may be improved by using alternate reporter constructs in which the *loxP* sites are positioned closer together, thus facilitating recombination (183).

Genetic lineage tracing does not require prior knowledge of which markers are expressed by the output cells and, thus, enables unbiased monitoring of cell ontogeny. Therefore, we were able to identify CDP-derived cells in cell populations previously thought

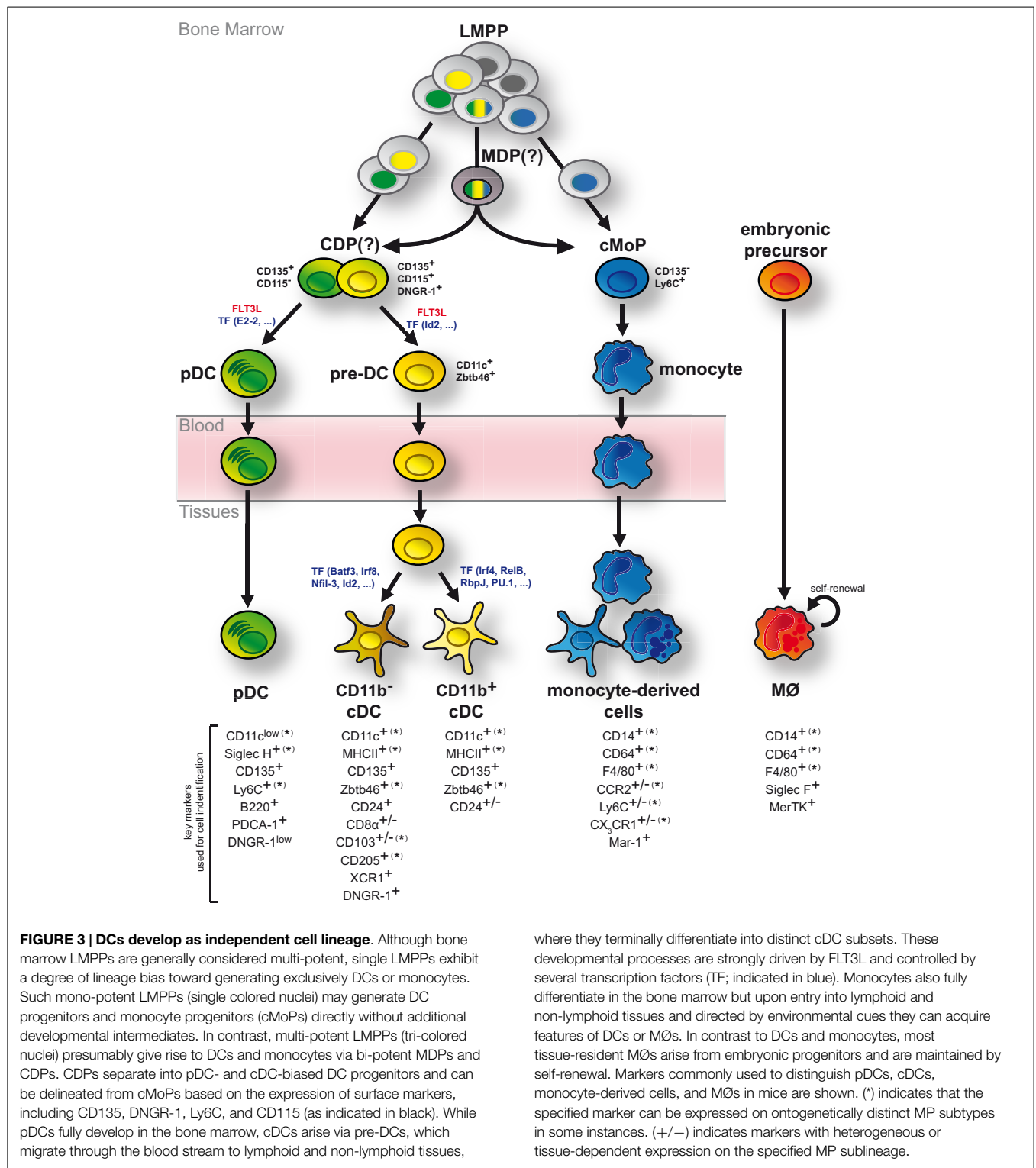
to constitute monocytes/MØs based on the expression of surface markers, such as CD64 (36). CD64<sup>+</sup> CDP-derived cells do not express *Clec9a* message and are especially frequent in kidneys, although the presence of few YFP<sup>+</sup> cells in the CD64<sup>+</sup> component of lung and small intestine indicates that atypical CDP-derived cells also exist in other tissues (36). CD64<sup>+</sup> kidney DCs resemble yolk sac-derived F4/80<sup>hi</sup> tissue-resident MØs, appear to lack *Zbtb46* expression (37) and their affiliation as DCs or MØs has been debated (184). We, therefore, used adoptive transfer as additional method to confirm cell ontogeny. Surprisingly, neither purified DNGR-1<sup>+</sup> CDPs nor total bone marrow generated F4/80<sup>hi</sup>CD64<sup>+</sup> CDP progeny in kidneys 1 week after adoptive transfer into irradiated recipients (36). Since kidney DCs reportedly have a slow turnover (185), it is possible that CDPs had insufficient opportunity to reach their renal niche and expand during short-term transfer experiments. Consistent with this notion, F4/80<sup>hi</sup>CD64<sup>+</sup> kidney leukocytes were efficiently generated from bone marrow progenitors in long-term reconstitution experiments (36). Therefore, our data strongly support a CDP origin of CD64<sup>+</sup> kidney leukocytes, despite their phenotypic resemblance to monocytes or MØs (36). These data exemplify the power of lineage tracing in following cell ontogeny in an unbiased way, although it is possible that DNGR-1 is expressed on yet unidentified developmental intermediates.

Addressing this possibility might require tamoxifen-inducible Cre constructs that can be used to pulse label progenitor populations (80). In the future, combinatorial approaches, such as “split-Cre” fragments controlled by two different promoters (186) or an intersection where Cre and the inducible reporter are driven by two cell-specific promoters (187, 188) may be of benefit to generate improved models to lineage trace DCs. The identification of CDP-derived cells with attributes of monocytes/MØs exemplifies the insufficiency of phenotypic properties, such as surface markers, as means of accurate cell identification of MPs. It also raises the question why cells of distinct ontogeny but overlapping phenotype exist in the same tissue. Further elucidation of the specific functions of MPs in immunity will benefit from lineage tracing approaches that result in target cell deletion through the use of inducible DTR or DT subunit modules (82, 112, 189, 190).

### Conclusion

The studies discussed above have significantly advanced our understanding of DC ontogeny but have also uncovered some uncertainties (Figure 3). While the bone marrow origin of DCs and monocytes is undisputed, the exact developmental intermediates and branching points between HSCs and DC progenitors remain to be clarified. Current data indicate that lineage imprinting toward DCs and monocytes may occur as early as LMPPs, potentially through epigenetic modification (Figure 3). This realization constitutes a major conceptual shift as it puts in question the existence of a bi-potential MDP and the resulting relatedness of DCs and monocytes. A definitive resolution of this question requires increasingly refined methods to genetically trace single progenitors or select DC and monocyte lineages. Nonetheless, it is clear that cDCs, pDCs, and monocytes can be separated based on their descent from committed





**FIGURE 3 | DCs develop as independent cell lineage.** Although bone marrow LMPPs are generally considered multi-potent, single LMPPs exhibit a degree of lineage bias toward generating exclusively DCs or monocytes. Such mono-potent LMPPs (single colored nuclei) may generate DC progenitors and monocyte progenitors (cMoPs) directly without additional developmental intermediates. In contrast, multi-potent LMPPs (tri-colored nuclei) presumably give rise to DCs and monocytes via bi-potent MDPs and CDPs. CDPs separate into pDC- and cDC-biased DC progenitors and can be delineated from cMoPs based on the expression of surface markers, including CD135, DNGR-1, Ly6C, and CD115 (as indicated in black). While pDCs fully develop in the bone marrow, cDCs arise via pre-DCs, which migrate through the blood stream to lymphoid and non-lymphoid tissues,

where they terminally differentiate into distinct cDC subsets. These developmental processes are strongly driven by FLT3L and controlled by several transcription factors (TF; indicated in blue). Monocytes also fully differentiate in the bone marrow but upon entry into lymphoid and non-lymphoid tissues and directed by environmental cues they can acquire features of DCs or MØs. In contrast to DCs and monocytes, most tissue-resident MØs arise from embryonic progenitors and are maintained by self-renewal. Markers commonly used to distinguish pDCs, cDCs, monocyte-derived cells, and MØs in mice are shown. (\*) indicates that the specified marker can be expressed on ontogenetically distinct MP subtypes in some instances. (+/-) indicates markers with heterogeneous or tissue-dependent expression on the specified MP sublineage.

developmental intermediates (Figure 3). Their differentiation is further driven by unique factors indicating that their developmental paths are distinct (Figure 3). In stark contrast to pDCs, cDCs, and monocytes, most tissue MØs arise from embryonic progenitors and are predominantly maintained by self-renewal into adulthood (Figure 3).

Taken together, these data unequivocally establish that DCs, monocytes, and MØs develop as unique cellular entities and although one could argue that most of this knowledge is derived from mouse studies, developmental parallels have been observed in other species (66–73). Despite these advances, we are at a loss for a universal definition of DCs that is readily accessible to

experts within and outside the field of MP biology. In light of this recognition, it has been suggested to revise the current nomenclature of MPs into a system that takes cell ontogeny into account when defining subpopulations (6). Such system would greatly aid our understanding of phagocyte biology as it remains uncertain to what extent the cellular origin of DCs, monocytes, and MØs determines the unique functionality of these cells in immunity and/or tissue homeostasis. While global profiling has revealed a role for the local tissue microenvironment in shaping the transcriptional landscape of DCs, monocytes, and MØs from different organs, certain gene signatures and transcriptional features are set by ontogeny (167, 191–193). Therefore, the full functional diversity of DCs, monocytes, and MØs is likely shaped by both nature (ontogeny) and nurture

(the environment). Since ontogeny is immutable it provides a more robust common denominator for cell definition that enables deciphering cellular functions without assuming preconceived functional or phenotypic relationships. DC classification based on cell ancestry is a work in progress but its implementation will ultimately yield a more robust and transparent way of cell definition.

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