Classical dendritic cells as a unique immune cell lineage

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Despite the critical role of classical dendritic cells (cDCs) in the initiation of adaptive immune responses, the genetic and phenotypic definition of cDCs remains moot. Two new studies designate Zbtb46 as a novel transcription factor that is specifically expressed in all cDCs in both humans and mice. Although Zbtb46 appears dispensable for cDC development, its specific pattern of expression supports the notion that cDCs constitute a unique immune cell lineage. Furthermore, these two studies provide novel tools that will aid in the study of cDC progenitors, visualization of cDCs in vivo, and depletion of cDCs for functional analysis.

DCs were originally defined by their characteristic dendritic morphology and extraordinary capacity for antigen presentation and T cell priming (Steinman 2012). These classical, or conventional, DCs (cDCs) are now classified into two main subsets, the $CD11b^+$ and $CD8^+$/$CD103^+$ cDCs in mice and the corresponding BDCA-1^+ and BDCA-3^+ cDCs in humans. Beyond these subsets, however, a significant functional, genetic, and phenotypic diversity of DCs has been recently appreciated (Collin et al., 2011; Hashimoto et al., 2011). Additional DC types include plasmacytoid DCs (pDCs), which are lymphocyte-like cells that specialize in type I interferon production; various tissue DCs that often display certain properties of macrophages; and several pathogen-induced populations such as TNF and inducible nitric oxide synthase (iNOS)–producing DCs (Tip-DCs), as well as monocyte-derived inflammatory DCs. Even within an apparently homogeneous cDC population such as splenic $CD11b^+$ cDCs, distinct subfractions are preferentially involved either in cytokine secretion or T cell priming (Lewis et al., 2011). This exciting variety brings forward a fundamental question: how does one define DCs in general and cDCs in particular?

Despite their nondendritic morphology, pDCs can be considered DCs based on their common origin and genetic similarity with cDCs (Reizis et al., 2011). However, distinguishing cDCs from other related cell types appears much more complex. Classical DC properties such as morphology, high MHC class II expression, and T cell priming capacity are informative but rather broad, difficult to assay in vivo, and can be affected by cell isolation. Phenotypic definitions can be misleading because they are often based on arbitrary surface markers. For example, the most specific murine cDC marker, CD11c, has no known function in cDCs and is highly expressed on some non-DCs such as alveolar macrophages. Thus, the commonly used definition of a cDC as any $CD11c^{high}$ MHC class II^+ cell is inadequate, and genetically relevant markers of the cDC lineage are urgently needed.

Zbtb46: a new and specific cDC marker

Two papers in this issue (see Meredith et al. and Satpathy et al.) describe a novel cDC-specific gene, Zbtb46 (also called zDC). Zbtb46 encodes a transcription factor of the BTB(POZ) family, and is expressed specifically in both human and murine cDCs and their committed progenitors. Indeed, Zbtb46 (under its alias Btbd4) was included in a cDC-specific gene expression signature that is conserved between species (Robbins et al., 2008). The expression of Zbtb46 is also found in erythroid progenitors and endothelium (Satpathy et al., 2012), but it is restricted to cDCs among mature hematopoietic cells.

Using a GFP knockin reporter for *Zbtb46* expression, Satpathy et al. (2012) undertook a broad survey of cell types affiliated with DC lineage. In addition to the two major cDC subsets in all organs, Zbtb46 was also expressed in monocyte-derived inflammatory DCs and a fraction of lymph node $CD169^+$ macrophages. Conversely, Tip-DCs were Zbtb46 negative, whereas $CD11b^+$ $CD103^-$ DCs in the lung and intestinal lamina propria appeared heterogeneous for Zbtb46 expression (Fig. 1). This pattern appears to correlate with the T cell priming capacity of the respective cell types, as described in multiple recent experiments (Hashimoto et al., 2011). Importantly, Meredith et al. (2012) confirmed cDC-specific expression of Zbtb46 protein using a newly developed monoclonal antibody. Thus, Zbtb46 expression can be used as a specific, evolutionarily conserved marker of cDC lineage that differentiates it from other related cell lineages.

The progenitors of cDCs

Satpathy et al. (2012) and Meredith et al. (2012) also investigated Zbtb46 expression in DC progenitors. All DCs and other mononuclear myeloid cells, such as monocytes, comprise a common branch of hematopoiesis that is distinct from both lymphoid and canonical (i.e. granulocytic) myeloid cell development (Geissmann et al., 2010). A key cellular stage of DC development is the clonogenic common DC progenitor (CDP) in the BM, which can give rise to cDCs and pDCs but no other cell types (Naik et al., 2007; Onai et al.,

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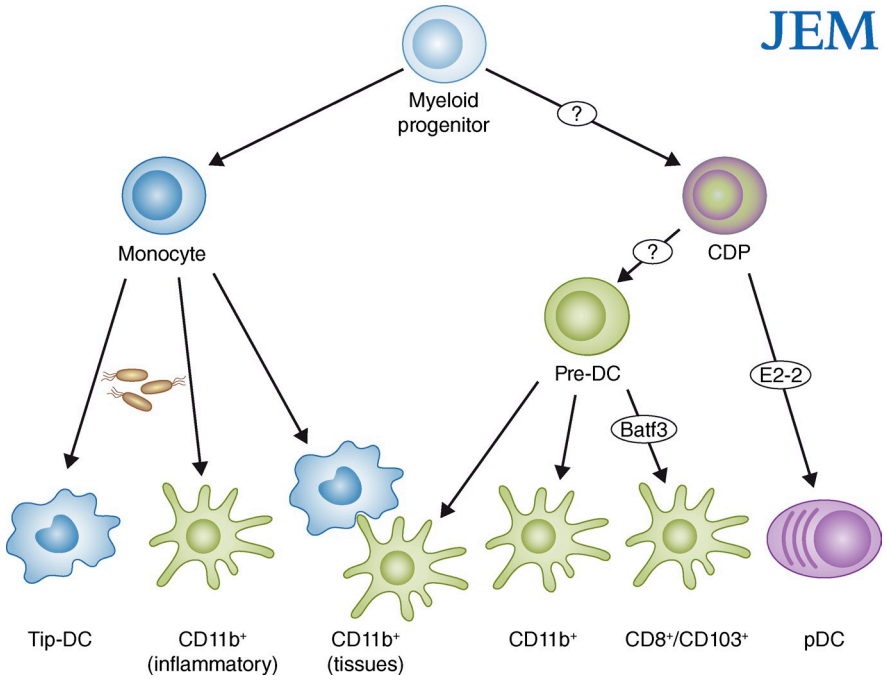

Figure 1. Specification of the dendritic cell lineage in the mouse. The DC progenitors and subsets that express Zbtb46 are shaded green. Note that the progenitors are represented as "conceptual" entities according to Zbtb46 expression rather than to the current phenotypic definitions. The known and possible (question marks) specific transcriptional regulators of DC development are indicated.

2007). The pDCs proceed to fully develop in the BM, whereas cDCs undergo terminal differentiation in the peripheral lymphoid organs or tissues. A clonogenic cDC-restricted progenitor (pre-DC) has been defined in the spleen (Naik et al., 2006), but it was unclear whether any earlier cDC progenitors existed in the BM. Liu et al., (2009) provided an affirmative answer by defining similar pre-DC populations in the BM and other tissues. However, unlike CDPs and splenic pre-DCs, BM pre-DCs have not been characterized in clonogenic assays, nor do they appear homogeneous by surface markers. Indeed, the proposed phenotype of pre-DCs overlapped with an immature pDC population identified in wild-type and E2-2–deficient BM (Cisse et al., 2008). Thus, the identity and mere existence of cDC-restricted progenitors in the BM had to be analyzed using more specific genetic markers.

This is where the *Zbtb46*-GFP knockin strain is likely to make an impact. Satpathy et al. (2012) demonstrate that CDPs, and especially pre-DCs, contain a distinct fraction of Zbtb46$^+$ cells, which can give rise only to cDCs but not pDCs in culture. In contrast, a major subpopulation of pre-DCs was shown to express the pDC marker SiglecH, and four distinct pre-DC populations could be defined by Zbtb46 versus SiglecH expression. The phenotypic definitions of CDPs and pre-DCs used by Satpathy et al. (2012) may need to be refined, and the clonogenic potential of the identified subpopulations remains to be established.

However, the results of Satpathy et al. (2012) emphasize two important points. First, cDC-committed progenitors do exist in the BM, confirming a "pre-commitment" to the cDC lineage at early stages of DC development as proposed previously (Liu et al., 2009). Of course, it is unclear whether such progenitors emigrate and give rise to cDCs in the periphery or produce the distinct BM-resident cDC population (Sapoznikov et al., 2008). Second, the currently used definitions of DC progenitors appear incomplete and should be revised based on genetic models such as the *Zbtb46*-GFP knockin reporter strain. Moreover, efficient ablation of Zbtb46-expressing pre-DCs, as described by Meredith et al. (2012), should facilitate their functional definition and reveal their potential in vivo. As in other lineages, DC progenitors likely represent a heterogeneous hierarchy of progressively diminishing developmental potentials rather than a linear sequence of distinct phenotypic populations.

Genetic control of the cDC lineage

Zbtb46 deletion did not affect murine DC development but resulted in lineage-inappropriate expression of several genes in cDCs (Satpathy et al., 2012). In addition, Zbtb46 facilitated cDC development when overexpressed (Satpathy et al., 2012). Additional consequences of Zbtb46 deletion (e.g. on cDC function) may eventually be discovered.

At present, it remains unclear what factors are specifically required for cDC development and, more broadly, for the development of all DCs (including cDCs and pDCs). Some transcription factors, such as PU.1 (Carotta et al., 2010) and Irf8 (Becker et al., 2012), are generally required for DC development at the level of DC progenitors, but their

effects are pleiotropic and not restricted to the DC lineage (Collin et al., 2011; Belz and Nutt 2012). More specific regulators of DC subsets include Batf3 for $CD8^+/CD103^+$ cDCs (Hildner et al., 2008) and E2-2 for pDCs (Cisse et al., 2008). In addition, certain signaling pathways such as Notch regulate DC subset development in a tissue-specific manner (Lewis et al., 2011).

It is possible that similarly specific transcriptional "master regulators" exist for all DCs as well as for all cDCs (Fig. 1). Alternatively, the specification of these lineages may be highly combinatorial, with multiple factors and signaling pathways contributing in an overlapping manner. In any case, the identification of a conserved cDC-specific transcription factor firmly establishes cDCs as a single distinct immune cell lineage, irrespective of their developmental and phenotypic heterogeneity. The identification of binding targets and regulatory elements of Zbtb46 should facilitate the study of genetic mechanisms controlling cDC lineage specification.

Visualization of the cDC lineage

In recent years, high-resolution immunochemistry and live cell microscopy have provided important insights into the anatomy and cellular dynamics of immune responses. One pressing experimental need is the ability to visualize and track endogenous cDC populations both in tissue sections and by intravital microscopy. Staining for cDC markers such as CD11c is rarely satisfying given the specificity problems described in the previous section. Several fluorescent reporters have been generated and proved useful for cDC analyses by intravital microscopy, such as the visualization of sessile cDC networks in lymphoid organs (Lindquist et al., 2004) and of cDC–T cell interactions during bacterial infection (Khanna et al., 2010). However, these strains harbor conventional transgenes driven by the CD11c promoter and thus display nonspecific and/or variegated reporter expression.

The *Zbtb46*-GFP knockin strain (Satpathy et al., 2012) represents a welcome step toward a faithful cDC reporter, as the cDCs in this strain can be identified in tissue sections by anti-GFP antibodies. The expression of GFP in endothelial cells complicates the analysis to some degree; however, these cells can be distinguished from cDCs by their morphology and/or additional markers or eliminated by using BM chimeras. It remains to be seen whether the level of GFP expression will permit live cDC detection by multiphoton microscopy. If it does not, bacterial artificial chromosome (BAC)–based transgenic approaches can be used to generate multicopy Zbtb46 transgenic reporter lines offering increased expression levels. Such next-generation reporters would greatly facilitate the analysis of cDC localization and function in vivo. Finally, the anti–Zbtb46 antibody developed by Meredith et al. (2012) may allow direct and specific visualization of cDCs by immunochemistry in any mouse strain.

Analysis of cDC lineage function by ablation

Just as T and/or B cell–deficient mice revolutionized the study of adaptive immunity, a diphtheria toxin (DT)–based system of cDC ablation greatly accelerated the analysis of cDC function (Jung et al., 2002). For instance, this DT-based system showed that cDCs are required for priming of alloreactive and antigen-specific T cells in the spleen, and thereby confirmed cDCs as the ultimate antigen-presenting cell type. However, the original CD11c promoter-based system appears insufficiently specific, as it mediates significant depletion of other cell types including splenic macrophages, monocytes, natural killer cells, and activated T cells (Jung et al., 2002; Probst et al., 2005). Furthermore, massive DT-induced cDC death has nonspecific effects such as functionally relevant neutrophil accumulation (Tittel et al., 2012). Finally, DT appears to affect additional DT receptor (DTR)–expressing nonhematopoietic cell types and kills mice if administered repeatedly. Thus, any unusual functions of cDCs demonstrated solely in the *CD11c*–DTR system must be evaluated with extreme caution.

Subsequent studies established additional systems of cDC ablation, including DTR expression from BAC-based CD11c transgenes (Tittel et al., 2012) and Cre recombinase-based binary systems (Birnberg et al., 2008). These systems provide improved specificity but have additional drawbacks such as myeloproliferation caused by cDC loss. The deletion of transcriptional regulators of DC development allows constitutive ablation of DC subsets (Hildner et al., 2008; Cervantes-Barragan et al., 2012); however, as discussed, a specific transcriptional regulator of all cDCs remains to be identified.

Meredith et al. (2012) took advantage of the exquisite cDC-specific expression of Zbtb46 to generate a novel cDC ablation system based on DTR knockin into *Zbtb46* locus. The problem of nonspecific DT sensitivity still exists in the resulting *Zbtb46*-DTR strain, probably as a result of Zbtb46 expression in the endothelium. This necessitates the use of BM chimeras and limits the utility of the strain, although a potential "therapeutic window" of DT administration to nonchimeric mice might eventually be found. Furthermore, DT-induced neutrophilia is also observed in this strain. However, parallel comparison with the original *CD11c*-DTR strain in BM chimeras revealed a greatly improved specificity of cDC ablation such that most non-cDC cell types remain unaffected.

Using this new Zbtb46-DTR strain, Meredith et al. (2012) further characterize the function of cDCs. Previous analysis of Batf3-deficient mice showed the importance of $CD8^+/CD103^+$ cDCs for the T cell–mediated control of the intracellular protozoan parasite *Toxoplasma gondii* and rejection of immunogenic sarcomas (Hildner et al., 2008; Mashayekhi et al., 2011). Similarly, DT-treated *Zbtb46*-DTR BM chimeras showed increased susceptibility to *T. gondii* and impaired rejection of antigen-expressing melanoma after vaccination. Importantly, the effects of *Zbtb46*-DTR–mediated ablation were significantly milder than the effects of *CD11c*-DTR–mediated ablation, consistent with the higher specificity of cDC depletion. These results emphasize the critical influence of lineage

specificity in depletion models and suggest that depletion models based on relevant and specific transcription factors should be used for the functional analysis of cDCs.

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