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Mechanisms regulating dendritic cell specification and development

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Summary

Understanding the diversification of dendritic cell (DC) lineages is one of the last frontiers in mapping the developmental hierarchy of the hematopoietic system. DCs are a vital link between the innate and adaptive immune responses, thus elucidating their developmental pathways is crucial for insight into the generation of natural immunity and for learning how to regulate DCs in clinical settings. DCs arise from hematopoietic stem cells through specialized progenitor subsets under the direction of FMS-like tyrosine kinase 3 ligand (Flt3L) and Flt3L receptor (Flt3) signaling. Recent studies have revealed important contributions from granulocyte-macrophage colony-stimulating factor (GM-CSF) and type I interferons (IFNs) *in vivo*. Furthermore, DC development is guided by lineage-restricted transcription factors such as IRF8, E2-2, and Batf3. A critical question centers on how cytokines and lineage-restricted transcription factors operate molecularly to direct DC diversification. Here we review recent findings that provide new insight into the DC developmental process.

Keywords

dendritic cells; cytokines; transcription factors; hematopoiesis; lineage commitment/specification

Introduction

Pathogen recognition, activation of immediate and long-term immunity and preserving tolerance to self-antigens are central to maintaining health. These key functions of the immune system are performed by dendritic cells (DCs), named for their unique morphology, which is characterized by dendrite-like extensions that mediate cell:cell contacts to regulate lymphocytes via antigen-presentation (1). In the nearly 40 years since the discovery of DCs as the major antigen-presenting cells (APCs) of the immune system by Steinman, Cohn, and colleagues (2–5), many DC subsets have been identified, and various regulatory events controlling their production and function have been elucidated. Gaps remain, however, in our understanding of the molecular mechanisms that dictate DC subset specification and development. Here we discuss recent advances in this area, focusing on the interplay between cytokines and transcription factors that control DC lineage development.

DC subsets

DCs are defined by cell surface marker protein expression, anatomical location, and functional responses. In mouse, three major types of DCs exist in steady state conditions, the plasmacytoid DCs (pDCs), resident lymphoid organ DCs (resident DCs), and peripheral tissue migratory DCs (migratory DCs) (reviewed in 1,6–8) (Fig. 1). As their name implies, resident DCs exist in lymphoid tissue, while migratory DCs are present in nonlymphoid tissues and transit to lymphoid organs upon activation. Infection or inflammation boosts DC numbers, although the ‘inflammatory DCs’ produced in these conditions do not fully resemble DCs found in steady state and utilize a distinct developmental pathway, and thus can be considered a separate class (9,10). Resident DCs, also termed ‘conventional DCs’, are defined in spleen by the surface phenotypes $CD11c^+CD11b^-$ major histocompatibility complex class II (MHC II) $^+CD205^+CD4^-CD8\alpha^+$ (resident spleen $CD8\alpha^+$ DCs) and $CD11c^+CD11b^+MHC\ II^+33D1^+CD4^+CD8\alpha^-$ (resident spleen $CD4^+CD8\alpha^-$ DCs) (6,11). A minor population of $CD11c^+CD11b^+CD4^-CD8\alpha^-$ DCs (resident spleen $CD11c^+CD11b^+$ DCs) is also observed. Resident spleen $CD8\alpha^+$ DCs are found in the T-cell zones and exhibit higher Toll-like receptor 3 (TLR3) expression, interleukin-12 (IL-12) production, MHC class I presentation, and cross-presentation activities versus other splenic DC subsets, while resident spleen $CD4^+CD8\alpha^-$ DCs are localized to marginal zones and exhibit stronger MHC class II presentation activity compared to other DC types (11–16, reviewed in 6,17). Subsets with similar marker characteristics and functional traits are found in lymph nodes (LNs), e.g. $CD11c^{hi}MHC\ II^+CD205^+CD8\alpha^+$ (resident LN $CD8\alpha^+$ DCs) and $CD11c^{hi}MHC\ II^+33D1^+CD8\alpha^-$ (resident LN $CD8\alpha^-$ DCs) (18,19). LNs also contain migratory DC populations that share certain markers with resident LN DCs (e.g. low $CD8\alpha$, $CD205$) (18) yet originate from the peripheral tissues, and thus the total LN DC repertoire is more extensive than that of spleen.

Migratory DCs, also referred to as ‘tissue DCs’, are positioned in several organs including skin, lung, and intestine. Skin contains epidermal Langerhans cells (LCs) and two dermal DC populations, $CD103^+langerin^+CD11b^{lo}$ (dermal $CD103^+$ DCs) and $CD11c^+CD11b^+CD103^-$ subsets (dermal $CD103^-$ DCs) (20–23, reviewed in 24,25) (Fig. 1). Dermal $CD103^+$ and $CD103^-$ DCs express distinct transcription factor profiles and utilize different developmental pathways, with dermal $CD103^+$ DCs being closely related to resident lymphoid organ $CD8\alpha^+$ DCs as well as $CD103^+$ DCs in other tissues including the lamina propria (LP) $CD103^+CD11b^-$ DCs (26). By contrast, dermal $CD103^-$ DCs appear to be a heterogeneous population (26,27). In LP, $CD103^-$ DCs were shown to descend from monocytes (28) (Fig. 1). Regardless of their developmental origin, migratory DCs share the properties of antigen uptake in peripheral tissues and migration via afferent vessels to the lymph nodes. Resident DCs, migratory DCs, and inflammatory DCs are inherently efficient antigen-presenting cells (APCs) capable of eliciting lymphocyte responses, including activation of $CD4^+$ T helper 1 (Th1) and Th2 cells (reviewed in 29–31). Lymphocyte responses are dependent upon the intrinsic antigen presentation machinery within DCs, which mediates antigen capture and regulated secretion of antigen-containing MHC I and II molecules to the DC cell surface (32), as well as production of an array of cytokines from activated DCs (e.g. IL-12, $IFN\gamma$) (29–31). Excellent reviews on DC subset localization, phenotype, and function have been published recently (10,24,33). From this point forward, we use the term ‘DC’ to refer to the collective assortment of resident and migratory DC subsets, while excluding reference to the pDC lineage, which is classified separately. Further, in some cases we delineate specific DC subsets by category (e.g. resident or migratory) and/or anatomical location where clear indications exist in the literature. As DC subsets are still being resolved and most studies have not been able to comprehensively investigate all DC populations, the information provided here is a snapshot of an emerging area of intense investigation.

Plasmacytoid DCs

By contrast with DCs, pDCs are the major type I interferon (IFN)-producing cells in the body and are considered to be a primary defense against viral pathogens (34–36) (reviewed in 8,37,38). Recently, pDCs have been implicated in wound healing (39), indicating a significant role in tissue repair processes. pDCs are activated by triggering of endosomal TLRs, e.g. TLR7 and TLR9, which recognize nucleic acids derived from infectious virus (34–36) or, in tissue injury and autoimmune conditions, host cellular debris (40,41). pDCs mature to an APC-like state following TLR triggering. The maturation process is characterized by downregulation of IFN secretion and induction of MHC II, costimulatory molecules, and cytokine production (42). In mouse, pDCs are defined by their surface phenotype of CD11c⁺CD11b[−]B220⁺PDCA1⁺SiglecH⁺, their plasma cell-like morphology, and their ability to rapidly (e.g. within 4 h) secrete abundant amounts of type I IFNs upon TLR triggering (35,43). pDCs reside in bone marrow, blood, thymus, and T-cell rich areas of lymphoid organs in steady state conditions and can be localized to skin and other tissues in inflammation or autoimmunity (36,44,45).

pDCs and DCs originate from hematopoietic stem cells (HSCs) (46) (Fig. 1), yet the factors that regulate lineage specification, commitment, and development are only now being defined. A major distinction occurs between migratory subsets that self-renew *in situ* in steady state conditions (e.g. epidermal DCs) (47), compared to pDCs and DC subsets that are continuously regenerated from bone marrow progenitors. A second bifurcation exists between DC subsets derived from monocytes (e.g. inflammatory DCs, LP CD103[−] DCs) and DCs that originate from specialized pDC/DC progenitor populations (28,48–51) (Fig. 1). These specialized pDC/DC progenitors arise within the lin[−]c-Kit⁺ progenitor population in the bone marrow (52), and proceed sequentially through stages termed the macrophage-DC progenitor (MDP), i.e. lin[−]c-Kit^{hi}CD115⁺CX3CR1⁺Flt3⁺ cells, and the common DC progenitor (CDP), i.e. lin[−]c-Kit^{int}CD115⁺Flt3⁺ cells (48,50,51,53). In this review, we focus on the developmental pathways of pDCs and DC populations that are replenished from dedicated pDC/DC bone marrow progenitors in steady state, while referring to recent reviews on locally renewing migratory DC subsets and monocyte-derived DC populations (25,54).

Cytokine control of pDC and DC generation

GM-CSF

DCs were first grown *in vitro* in blood or bone marrow cultures supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) (55,56); results suggested this important myeloid cytokine might regulate DC generation *in vivo*. Surprisingly however, genetic deletion of GM-CSF or the GM-CSF receptor β chain (GM-CSFR β) results in only a modest decrease in resident DCs in steady state conditions (57,58). Since GM-CSFR β is required for IL-3 and IL-5 signaling, these data also indicate that development of resident DCs is largely independent of IL-3 and IL-5 function *in vivo*. Migratory DCs in the skin are also reduced in GM-CSF-null mice but not completely ablated (57,59), indicating a partial requirement for GM-CSF. By contrast, overexpression of GM-CSF in transgenic animals or mice receiving daily injections of a modified form of recombinant GM-CSF designed to increase serum half-life showed a significant expansion in DC amounts in spleen and thymus (58,60,61), with the expanded DC populations most likely representing inflammatory DCs. It is now evident that GM-CSF drives inflammatory DC development from monocytes (49) (Fig. 1), and this monocyte-DC pathway can be observed *in vivo* in GM-CSF-dependent inflammatory conditions or thioglycollate-induced peritonitis (62,63) (Fig. 1). GM-CSF-driven inflammatory DCs resemble a tumor necrosis factor- α (TNF α)- and inducible nitric oxide synthase (iNOS)-producing DC subset (Tip DCs) that appears

during *L. monocytogenes* infection (64); however, neither inflammatory DCs nor Tip DCs appear to correspond to DC subsets found in steady state (62). As GM-CSF amounts are increased naturally during infection or inflammation (65), the production of inflammatory DCs from monocytes and potentially other precursor subsets may serve as an important means to increase APC function during conditions of physiologic demand. For purposes of developmental and functional DC studies, however, it is important to keep in mind that DCs derived *ex vivo* in GM-CSF cultures most likely represent DCs formed during inflammatory but not homeostatic conditions.

Flt3 ligand

FMS-like tyrosine kinase 3 ligand (Flt3L) and its receptor Flt3, a member of the tyrosine-kinase receptor family, comprise the major extracellular signaling pathway regulating steady state pDC and DC generation from bone marrow progenitors *in vivo* (57,66) (Fig. 1). The unique role of Flt3L versus GM-CSF in the differentiation of both pDCs and DCs from hematopoietic progenitor cells was first recognized in *ex vivo* human bone marrow cultures (67) and was later confirmed in mouse bone marrow cultures (68). The importance of Flt3L *in vivo* was established by injection of the recombinant cytokine into mice, which stimulates a massive expansion of pDCs and resident DCs in spleen (60,61,69,70). Conditional expression of Flt3L in transgenic mice expressing a tetracycline-inducible *Flt3l* cDNA expands DC amounts in blood, bone marrow, lymphoid organs (e.g. spleen, thymus, lymph node) as well as other tissues including liver and intestine (71), confirming that Flt3L provides an important stimulus for resident DC production *in vivo* and indicating its importance in migratory DC generation. Correspondingly, *Flt3l*^{-/-} or *Flt3*^{-/-} mice show reduced amounts of resident DCs and dermal DCs under homeostatic conditions (57,66,72). Subsequent studies have shown Flt3L treatment induces MDP numbers approximately 10-fold in bone marrow (72), indicating cytokine-sensitivity at an early progenitor stage. Moreover, in steady state, Flt3L and Flt3 control the abundance of the MDP in bone marrow (57,72), although MDP amounts are not completely ablated in the absence of Flt3L-Flt3 signaling (30–40% reduction), indicating redundant pathways support MDP maintenance. In addition, homeostatic regulation of DC precursors in the spleen requires Flt3 (72,73). Collectively, these results indicate that supraphysiological amounts of Flt3L expand early pDC/DC progenitors and promote pDC and DC production from the bone marrow compartment, while in steady state conditions Flt3 signals maintain bone marrow MDPs and resident DC numbers (Fig. 1). Studies with mice deficient in both GM-CSF (CSF2) and Flt3L (i.e. *Csf2*^{-/-} *Flt3l*^{-/-} mice) show that GM-CSF is also involved in regulating CDPs, resident DCs, and dermal DCs (57); however, as MDPs, pDCs, and DCs are still detected in *Csf2*^{-/-} *Flt3l*^{-/-} mice, these data also suggest the existence of additional factors that control pDC/DC progenitor maintenance as well as pDC and DC generation. One such factor appears to be M-CSF, which is able to stimulate pDC and DC development from bone marrow progenitors *in vitro* in the absence of Flt3L signaling. *In vivo*, M-CSF treatment induces pDC and resident spleen DC generation in mice lacking Flt3L (74); however, the development of resident lymphoid organ DCs in homeostatic conditions does not appear to require this cytokine. M-CSF is also important for certain migratory DC subsets, including CD103⁺ DCs in gut and peripheral tissues, and Langerhans cells in skin (26,28,75) (Fig. 1). Whether M-CSF serves a redundant function in the generation of resident DCs in the absence of both GM-CSF and Flt3L signaling requires examination of DC amounts in mice that lack all three growth factors.

The availability of Flt3L in recombinant form has enabled *ex vivo* expansion of pDCs and DCs from bone marrow or spleen in tissue culture (68,76–78), facilitating developmental and functional studies. Importantly, DCs that develop in Flt3L cultures (Flt3L-generated DCs) appear to correspond to resident DC subsets that are present in spleen under

homeostatic conditions (76), in contrast to the inflammatory DCs formed in GM-CSF cultures. While *in vitro* Flt3L-generated DCs lack the full repertoire of resident spleen DC marker proteins (e.g. deficient in CD8 α), expression and functional assays solidified the relationship between steady state spleen DC subsets and DC populations generated in Flt3L cultures *in vitro* by demonstrating close similarity in transcription factor and cell surface marker profiles, cytokine secretion patterns, and efficient MHC class I-mediated antigen presentation by classical and cross-presentation routes (76). Flt3L cultures have also led to the identification of spleen DC precursors (62), which are capable of generating resident spleen CD8 α ⁺ DCs and CD4⁺CD8 α ⁻ DCs. Moreover, pDCs produced in bone marrow cultures with Flt3L show a potent type I IFN response upon TLR triggering by CpGA (TLR9 triggering) or viral infection, indicating functional equivalence to pDCs produced *in vivo* under homeostatic conditions (68).

GM-CSF has the interesting property of suppressing Flt3L-dependent pDC generation in bone marrow cultures (68). Initially, it was not clear if GM-CSF induced pDC apoptosis or blocked pDC maturation. We found that the inhibitory effect of GM-CSF is exerted at an early stage in the pDC developmental pathway. Using purified bone marrow progenitors, we showed that GM-CSF dominantly inhibits Flt3L-driven pDC development from the lin⁻Flt3⁺ bone marrow compartment *in vitro* (79) (Fig. 1). Furthermore, pDC numbers are decreased by ~75% in bone marrow and ~40% in spleen in response to upregulated GM-CSF expression mediated by hydrodynamic gene transfer (HGT) (Li H, Gelbard, A, Martinez, GJ, Esashi, E, Zhang, H, Ngyuen-Jackson, H., Liu, YJ, Overwijk, WW and Watowich, SS, unpublished results). Since steady state pDC numbers are unaffected in *Csf2*^{-/-} mice relative to wildtype animals (57), these studies are consistent with a role for GM-CSF in demand but not homeostatic conditions. The resulting effect of GM-CSF induction is a dramatic skewing of the pDC to DC/inflammatory DC ratio, i.e. ~1:5 vs. 1:27 pDC:DC/inflammatory DC in homeostatic conditions versus GM-CSF HGT (Li *et al.*, unpublished results). While this reduction in pDCs upon GM-CSF treatment might be expected to increase susceptibility to viral infection, patients receiving GM-CSF therapy do not appear to suffer more frequent or potent viral infections (80). We speculate that the response to GM-CSF may be in place to dampen innate production of IFN- α via pDCs, as type I IFNs can strikingly reduce DC numbers in certain settings (81) (Fig. 2).

Type I IFNs

Measles virus (MV) or lymphocytic choriomeningitis viral infection was shown to suppress DC development via type I IFN signaling (i.e. IFNAR-dependent), indicating a novel immune-evading mechanism utilized by these pathogens (81). Both GM-CSF-driven DC development *in vitro* and Flt3L-driven generation of splenic CD8 α ⁺ DCs *in vivo* was blocked by viral infection, suggesting that formation of inflammatory DCs as well as resident spleen CD8 α ⁺ DCs is sensitive to type I IFN signaling (81). This idea was confirmed by data showing a decrease in total splenic CD11c⁺ cells following IFN- β treatment *in vivo* (81). By contrast, neonatal mice infected with an attenuated strain of vaccinia showed induction of serum Flt3L, a corresponding rise in resident spleen DCs and pDCs, and enhanced defense against heterologous viral infection; these responses were dependent upon an intact IFN system *in vivo* (82). Furthermore, in studies to determine if the suppressive action of GM-CSF on pDC development could be negated, we identified IFN- α and IFN- β as acting to promote the generation of cells that show many phenotypic features of pDCs except the ability to produce type I IFN upon TLR9 triggering (Li *et al.*, unpublished results). IFN- α HGT stimulates the accrual of cells resembling pDCs *in vivo*, while simultaneously reducing DC numbers in spleen and bone marrow (Li *et al.*, unpublished results) (Fig. 2). Thus, type I IFNs appear to have distinct effects on pDC and DC development, namely induction of pDC numbers and general suppression of DCs.

However, the negative effects of type I IFNs on DCs does not extend to all conditions (82), suggesting the function of type I IFNs *in vivo* may rely in part upon the specific cytokine profile in the infected animal and/or the age or developmental stage of the host. As different pathogens are likely to induce distinct cytokine profiles within specific microenvironments, we suggest that unique cytokine milieus that arise during infection may tailor local and/or systemic pDC vs. DC ratios to manage pathogen-specific host immune responses. However, this topic is relatively understudied and additional work is necessary to clarify the relevance of local and systemic cytokine production during infection and its effect upon pDC and DC generation as well as pDC/DC-regulated immune responses. Studies that utilize important human pathogens, along with natural dosages and routes of delivery, will be particularly valuable in elucidating why viral infections and type I IFNs regulate DC and pDC abundance *in vivo*.

To understand the functional consequence of IFN- α on pDCs, we analyzed cells generated in response to IFN- α *in vitro* or *in vivo*; these studies showed that IFN- α -elicited pDCs express the full repertoire of pDC cell surface marker proteins (i.e.

CD11c⁺CD11b⁻B220⁺PDCA1⁺Siglec H⁺) and exhibit the unique pDC ultrastructural morphology, characterized by a well-developed endoplasmic reticulum (35, Li *et al.*, unpublished results), and therefore closely resemble traditional Flt3L-generated pDCs. However, the IFN- α -elicited pDC population lacks TLR-triggered type I IFN production, and thus the cells do not directly correspond to pDCs. We refer to this population as IFN-pDCs to distinguish them from classical pDCs and to reflect the fact that they have been generated or 'conditioned' under the guidance of IFN- α . At this point, it is unclear whether IFN-pDCs are a discrete subset that is closely related to pDCs or if they represent a distinct developmental stage of the pDC lineage (e.g. functionally arrested or further differentiated). Importantly, cells with the characteristics of IFN-pDCs (e.g. pDC marker profile, deficiency in type I IFN production) were upregulated during vesicular stomatitis virus (VSV) infection, which is accompanied by high levels of endogenous IFN- α production. By contrast, VSV infection suppressed bone marrow and spleen DC numbers (Li *et al.*, unpublished results). These data suggest that VSV-induced IFN- α may stimulate a feedback response to the bone marrow to boost production of IFN-pDCs and simultaneously inhibit DC generation and/or maintenance. As IFN-pDCs are efficient APCs (Li *et al.*, unpublished results), their induction may compensate for the loss of DCs during VSV infection. Interestingly, the response to VSV resembles the increase in pDCs observed during infection with attenuated vaccinia virus (82); however, VSV and vaccinia have distinct effects on DC generation (suppressive vs. stimulatory, respectively). These results further support the idea that cytokine profiles and/or developmental stage of the host is likely to factor significantly in the response of pDCs and DCs during infection. IFN- α is traditionally considered to be an inflammatory, anti-viral, and anti-proliferative factor, due to its well-documented effects including induction of host genes that interfere with viral replication and cellular proliferation (83), thus induction of IFN-pDCs by this cytokine was somewhat unexpected. Recently, however, IFN- α was shown to stimulate HSC proliferation through direct and indirect mechanisms, indicating it elicits intracellular and extracellular signals to drive dormant HSCs into cell cycle (84). This pathway may contribute to the generation of IFN-pDCs *in vivo*.

Origins of pDCs and DCs

The importance of Flt3L-Flt3 signals in pDC and DC production led to the identification of a lin⁻Flt3⁺ bone marrow progenitor population, which is capable of generating pDCs and resident DCs following adoptive transfer into lethally irradiated hosts (52). These studies provided initial evidence for a specialized bone marrow progenitor of pDC and DCs. Subsequent refinement of progenitor populations identified MDPs, which give rise to

monocyte-macrophage-lineage cells, DCs and pDCs (48,53), and the more restricted and developmentally successive CDP subset (48,50,51), which generates pDCs and DCs but not monocyte/macrophage lineage cells (Fig. 1). Progenitors with restricted developmental potential for lymphoid or myeloid lineages [the common lymphoid progenitor (CLP) or common myeloid progenitor (CMP)] can also generate pDCs and resident DCs *in vivo*, although DC generation is restricted to the Flt3⁺ subset of the CLP and CMP populations (52,85). Fully developed pDCs are found within the bone marrow; however, a relative paucity of DCs is observed, supporting the notion that DC differentiation involves the release of a DC-restricted progenitor from the marrow. This concept was verified by the identification of DC precursors in spleen (62) as well as studies using mice with experimentally manipulated shared circulatory systems (parabiotic mice), which showed that resident DCs are continuously regenerated by a short-lived circulating DC progenitor, the pre-DC (72,86). Pre-DCs are localized in bone marrow, blood, and lymphoid organs (48) and have recently been shown to generate dermal CD103⁺ DCs in addition to resident DCs (26). While a restricted pDC progenitor has yet to be defined, the existence of such would be consistent with successive lineage restriction events that govern hematopoiesis. Thus, commitment to the pDC and DC lineages occurs within the bone marrow compartment, involving specification events and, potentially, differential retention (pDC) or migration (pre-DC) signals that remain poorly understood.

Molecular cues regulating DC and pDC lineage formation

Lineage-regulatory transcription factors

Cell fate decisions in the hematopoietic system involve the actions of a small number of regulatory transcription factors, which establish specification in pluripotent progenitors and induce commitment and differentiation to the blood/immune cell lineages (87–91). Hematopoietic cell specification and lineage commitment is accomplished in part by the induction and maintenance of transcriptional autoregulatory loops that sustain the expression of a particular factor (89,91). This permits transcriptional programs that depend upon the autoregulated factor to be enacted to drive commitment and differentiation. Antagonistic interactions can occur among regulatory transcription factors; these often function to reject alternative cell fates (89,91). Moreover, precise control of transcription factor expression or the activity level of a given factor is a crucial part of hematopoietic lineage decisions, due to autoregulatory or opposing actions, or because regulators for a particular cell type operate in conjunction with one another to affect lineage-specific gene expression.

Insight into the mechanisms controlling pDC and DC lineage specification has benefitted from our understanding of these general concepts governing hematopoiesis. For example, transcription factor expression patterns in pDCs or DCs have accurately predicted roles in their developmental decisions (92–95). Overall, however, our knowledge of pDC/DC transcriptional control mechanisms is less refined than those guiding development of T and B lymphocytes or the major myeloid cell types such as monocytes, granulocytes, erythrocytes, and megakaryocytes. One reason for this difference is the relatively recent identification of DCs and pDCs, compared to other blood cell lineages, coupled with the fact that many DC subsets are still being characterized at phenotypic and functional levels, and developmental relationships between DCs subsets remain poorly understood. Moreover, the diversity of DCs, the similarity between DCs and monocytes, and monocyte-DC conversion complicated analyses. The advent of molecular profiling, *in vivo* reporters and advanced anatomical studies has and will continue to distinguish DC subsets, enable their molecular characterization and ultimately provide a more complete understanding of the transcriptional networks regulating their development.

At this juncture, it appears that factors controlling pDC and DC production *in vivo* fall into roughly two classes: (i) those that function broadly across several DC lineages and pDCs as well as myeloid or lymphoid cell types (e.g. PU.1, Gfi1, Ikaros) (96–102) and thus are likely to regulate multipotent and/or pDC/DC progenitor (e.g. MDP or CDP) maintenance and/or differentiation potential, versus (ii) transcription factors that have specific roles in one or more (but not all) of the DC lineages and/or in pDCs. Factors in the latter category include the basic helix-loop-helix (bHLH) transcription factor (or E protein) E2-2/Tcf4, which is exclusively required for pDC development but not other blood lineages (95), and the AP-1-like protein Batf3, which controls resident CD8 α ⁺ DCs and dermal CD103⁺ DCs but does not regulate other DC subsets (27,94). Here we focus on transcriptional control of the pDC and resident CD8 α ⁺ DC subsets, as these lineages have specific (e.g. E2-2 and Batf3) as well as overlapping [e.g. interferon response factor (IRF8), inhibitor of differentiation/DNA binding protein 2 (Id2)] transcription factor requirements and may therefore provide a paradigm for understanding mechanisms of pDC/DC subset specification and commitment.

E2-2

E2-2/Tcf4 expression is highly restricted within the hematopoietic system to pDCs, and analysis of E2-2-deficient mice confirmed an essential role for E2-2 in pDC generation *in vivo* (95) (Fig. 3). Since *Tcf4*^{-/-} animals die at birth, *Tcf4*^{-/-} fetal liver progenitors were used in bone marrow transplantation experiments to examine E2-2 function. These studies showed that *Tcf4*^{-/-} progenitors fail to reconstitute pDCs in bone marrow or spleen; however, other blood lineages were formed in normal abundance (95). Animals with conditional *Tcf4* deletion have a specific loss of pDCs but not resident DCs or other immune subsets (e.g. B cells, T cells, macrophages) (95). Moreover, E2-2 deficiency results in the accumulation of a bone marrow population that expresses some but not all pDC marker proteins (i.e. CD11c⁺Ly-6C⁺PDCA1⁻ cells), suggesting accrual of a progenitor subset that fails to undergo terminal differentiation. The CD11c⁺Ly-6C⁺PDCA1⁻ progenitor population is observed in bone marrow from wildtype animals, although at reduced amounts relative to *Tcf4*^{-/-} chimeras (95), implying E2-2 deficiency prevents pDC maturation from a lineage-committed progenitor. These results are interesting, as they point to a possible pre-pDC population (CD11c⁺Ly-6C⁺PDCA1⁻ cells) and, furthermore, as they suggest E2-2 may not be necessary for specification of the pDC lineage from multipotent progenitors. If E2-2 were dispensable for pDC specification, this would indicate that other factors dictate pDC cell fate in multipotent progenitors such as MDPs or CDPs. Further analysis of the role of E2-2 in hematopoietic progenitor subsets, as well as studies that assess developmental options of the putative pre-pDC subset (CD11c⁺Ly-6C⁺PDCA1⁻ cells), is required to understand whether E2-2 is involved in pDC specification. Regardless, E2-2 has an essential and non-redundant role in guiding terminal pDC differentiation and in establishing pDC functional responsiveness. Precise E2-2 expression levels are crucial, as indicated by *Tcf4* haploinsufficiency for pDC production and IFN secretion (95). Humans with Pitt-Hopkins syndrome, who contain only one wildtype *Tcf4* allele, also show *Tcf4* haploinsufficiency, as pDCs isolated from these individuals are unable to produce type I IFN in response to nucleic acid or viral challenge. In mice and presumably humans, E2-2 directly regulates genes that are necessary for pDC function, including those encoding IRF7, TLR7, and TLR9. Interestingly, E2-2 also appears to control other pDC-related transcription factors such as SpiB and IRF8 (95). Thus, E2-2 is an essential component of the pDC transcriptional network and may be involved at multiple stages of pDC lineage development, including initiation/reinforcement of commitment, by regulating pDC-related transcription factors, and terminal differentiation, by controlling genes that are required for pDC functional responses.

Id2

The Id proteins Id2 and Id3 are established antagonists of bHLH E proteins such as E2-2 (103). Id proteins contain an HLH domain that binds the bHLH domain of E proteins, forming non-functional dimers that are unable to bind DNA. Id2 is an interaction partner for E2-2 (104,105). Accordingly, Id2 deletion augments pDC amounts approximately 1.5-fold *in vivo* and enhances pDC IFN production 2–4 times, relative to IFN secretion from wildtype pDCs (93) (Fig. 3). Thus, Id2 is a natural antagonist for the pDC lineage and appears to function in direct opposition to E2-2, i.e. Id2 inhibits pDC generation and IFN secretion while E2-2 orchestrates pDC development and IFN production.

The effects of Id2 on pDC production suggest Id2 has a role in suppressing E2-2 activity in pDC/DC progenitors, hence rejecting and/or limiting commitment to the pDC lineage. In agreement with this notion, Id2 is necessary for formation of resident CD8 α ⁺ DCs in spleen and dermal CD103⁺ DCs *in vivo* (Fig. 3). Thus, E2-2 antagonism via Id2 might be required for specification and/or commitment to these resident and migratory DC subsets. Total splenic CD11c⁺ numbers are equivalent between wildtype and *Id2*^{-/-} mice, however, and overrepresentation of splenic CD4⁺CD8 α ⁻ DCs appears to compensate for loss of splenic CD8 α ⁺ DCs in *Id2*^{-/-} animals (93). These results suggest that Id2 may also regulate lineage commitment decisions in spleen DC precursors, between CD8 α ⁺ DC and CD4⁺CD8 α ⁻ DC fates, in addition to decisions in a shared pDC/DC progenitor (e.g. CDPs). Alternatively, insufficient generation of resident spleen CD8 α ⁺ DCs in *Id2*^{-/-} animals may allow expanded colonization by splenic CD4⁺CD8 α ⁻ DCs and/or pDCs in niches that are normally occupied by CD8 α ⁺ DCs, via *in situ* proliferation of splenic DC precursors, increased influx/retention of bone marrow precursors and/or enhanced repopulation by differentiated pDCs. Regardless of which mechanism controls splenic DC subset proportions, the regulatory pathways that affect Id2 expression in pDC/DC multipotent progenitors would be expected to have key roles in modulating their transcriptional and developmental programs. Signals inducing Id2 may enable/favor resident spleen CD8 α ⁺ DC and dermal CD103⁺ DC generation and consequently limit pDC output from common pDC/DC progenitors (e.g. MDPs, CDPs), while upregulation of Id2 expression in spleen precursors may induce resident spleen CD8 α ⁺ DC development at the expense of splenic CD4⁺CD8 α ⁻ DCs. These developmental outcomes could be elicited by direct restraint of transcriptional programs that support pDC commitment (e.g. via inhibition of E2-2 function) and/or resident spleen CD4⁺CD8 α ⁻ DC generation (via an unknown factor), thus allowing activation of gene expression patterns that reinforce commitment to the resident spleen CD8 α ⁺ DCs and dermal CD103⁺ DC subsets. Elucidating the signals that regulate Id2 expression is of great interest; in addition, the mechanisms by which Id2 controls pDC/DC lineage decisions are important and will be clarified by determining a precise roadmap of DC precursor-product relationships, and a deeper understanding Id2 function in pDC/DC progenitor developmental potential.

IRF8

Unlike Id2, IRF8 is required for pDC, resident spleen CD8 α ⁺ DC, and migratory CD103⁺ DC development (Fig. 3), as these subsets are depleted in *Irif8*^{-/-} mice (26,106–108). Myeloid differentiation is also dependent upon IRF8, and *Irif8*^{-/-} animals develop a myeloproliferative/pre-leukemic phenotype characterized by excessive granulocyte production and failure to generate adequate monocyte numbers (109). A spontaneous point mutation (R294C) of IRF8 in BHX2 mice also causes myeloproliferative disease and, interestingly, affects resident spleen CD8 α ⁺ DC development and IL-12 production without impairing pDC generation (110,111). IRF8 residue 294 is located within the IRF association domain, which mediates protein:protein interactions, and the R294C mutation abolishes IRF8-partner-dependent DNA binding *in vitro* and *in vivo*. For example, the interaction

between IRF8 and PU.1 is lost with the R294C mutation, resulting in a failure to recruit IRF8 to the endogenous target gene cystatin C *in vivo* (111). Since pDC development remains intact in BHX2 animals, these results imply that IRF8 utilizes distinct partner proteins in pDCs versus resident spleen CD8 α ⁺ DCs (111). The identity of IRF8 partner proteins is of great interest for further understanding the molecular regulation of the pDC and resident spleen CD8 α ⁺ DC developmental programs.

The loss of monocytes at the expense of granulocytes and additional hematopoietic alterations including excess erythropoiesis in *Irf8*^{-/-} mice was originally interpreted as a role for IRF8 in a multipotent progenitor subset (109). The recent identification of a progenitor subset with monocyte, DC, and pDC developmental potential (MDPs) (48,53) suggests that IRF8 may be crucial for MDP maintenance and/or lineage specification from the MDP. Importantly, the abundance of CLPs and CMPs was normal in *Irf8*^{-/-} animals (92), while MDP or CDP amounts have not been determined on the IRF8-deficient background to our knowledge. Studies investigating progenitor amounts in *Irf8*^{-/-} mice as well as the developmental potential of *Irf8*^{-/-} progenitor subsets are necessary for a complete picture of IRF8 function during pDC/DC lineage commitment.

In addition to regulating pDC and DC subset generation *in vivo*, IRF8 also controls genes that have important roles in pDC and resident spleen CD8 α ⁺ DC functional responses, including TLR9 expression and type I IFN production in pDCs, and IL-12 production from CD8 α ⁺ DCs (112–115). These data indicate IRF8 transcriptional activity is necessary in terminally differentiated pDCs and resident CD8 α ⁺ DCs. Since IRF8 and E2-2 both participate in pDC differentiation, it is possible that these factors work cooperatively to regulate pDC-specific genes. In resident spleen CD8 α ⁺ DCs, IRF8 appears to collaborate with PU.1 (111), and this may extend to dermal CD103⁺ DCs, given their functional and developmental similarities with resident CD8 α ⁺ DCs. IRF2 has also been reported as an IRF8 binding partner (111); however, IRF8 and IRF2 control different resident spleen DC subsets, i.e., IRF2 is required for spleen CD4⁺CD8 α ⁻ DCs, while IRF8 regulates generation of CD8 α ⁺ DCs (92,106,116), indicating that IRF2:IRF8 binding complexes, if observed *in vivo*, are not likely to have essential function in the formation of resident spleen DC subsets.

Spi-B

The Ets family member Spi-B has an important role in pDC development from human pDC/DC progenitors (117) (Fig. 3). Spi-B has been reported to bind together with IRF8 on composite oligonucleotides containing ISRE and Ets/IRF elements using *in vitro* systems (111), although it is not clear if this interaction is found at endogenous pDC gene promoters *in vivo*. *Spib*^{-/-} mice show normal T and B cell amounts but impaired B-cell function and defective T-cell-dependent humoral immune responses (118), while to our knowledge pDC and DC populations have not yet been examined in *Spib*^{-/-} mice. *Spib*^{-/-} lymphocytes have cell autonomous defects; however, the impaired lymphocyte responses in *Spib*^{-/-} mice *in vivo* may also imply an underlying pDC and/or DC deficiency, in accord with the role for Spi-B in human pDCs (117). This possibility can be addressed directly by characterization of DC subset representation and function in *Spib*^{-/-} mice. The established role for Spi-B in human pDCs is significant, since our understanding of human DC generation is much less sophisticated relative to murine counterparts. Importantly, human pDCs and mouse pDCs share a requirement for E2-2 (95) and potentially Spi-B, suggesting close similarity between the human and murine pDC developmental pathways.

Batf3

The basic leucine zipper transcription factor Batf3 was the first factor identified with an exclusive role in a resident spleen DC subset. Batf3 has been reported to heterodimerize

with c-Jun and function as a transcriptional repressor for IL-2 production (119), although its molecular target(s) in DCs remains unknown. Batf3 is necessary for development of resident lymphoid organ CD8 α^+ DCs and dermal CD103 $^+$ DCs but not pDCs or resident lymphoid organ CD8 α^- DC subsets (27,94) (Fig. 3). Importantly, the selective effect of Batf3 deletion allowed for functional analysis of lymphoid tissue CD8 α^+ DCs. Splenic DCs isolated from *Batf3* $^{-/-}$ mice were unable to mediate antigen cross-presentation, demonstrating conclusively that resident CD8 α^+ DCs perform MHC class I presentation of exogenous antigen (94). Moreover, *Batf3* $^{-/-}$ mice lack virus-specific CD8 $^+$ T-cell responses and show defective rejection of immunogenic tumors, suggesting antigen cross-presentation by resident CD8 α^+ DCs elicits CD8 $^+$ cytotoxic T-cell responses to virus and tumors (94). However, the generation of migratory CD103 $^+$ DCs that populate several tissues including lung, intestine, and dermis is also dependent upon Batf3 (27). These cells have established roles in the cross-presentation of exogenous or cell-associated antigens and thus are also likely to mediate anti-viral and anti-tumor cytotoxic T-cell responses *in vivo*. Furthermore, the shared requirement for Batf3 between migratory CD103 $^+$ DCs and resident lymphoid organ CD8 α^+ DCs *in vivo* suggested these populations have a common developmental origin. This concept was recently bolstered by the observation that migratory CD103 $^+$ DCs are generated from circulating DC precursors that give rise to resident lymphoid organ CD8 α^+ DCs, as well as the fact that both DC populations require IRF8 and Id2 for efficient development *in vivo* (26,27) (Fig. 3). Overall, studies of DC transcriptional regulators have provided important insights that have enabled developmental relationships between resident and migratory DC subsets to be further elucidated, although in general knowledge in this area is limited. Further analysis of factors participating in the DC and pDC transcriptional networks should contribute toward a better understanding of pDC/DC developmental origins and lineage associations.

STAT function in DC and pDC developmental pathways

For pDCs and DCs as well as other hematopoietic lineages, a key question centers on the mechanisms that control the expression and/or activity of lineage-specific transcriptional regulators. Our attention has focused on cytokine signaling pathways, with the question of how they might influence transcription factor expression during pDC and DC development. Cytokines elicit multiple intracellular signaling cascades, and signal transducers and activators of transcriptions (STATs) are considered to be principal mediators of cytokine signal transduction. STATs are triggered by tyrosine phosphorylation, which is activated by cytokine receptor-associated Jak kinases following cytokine engagement. Tyrosine phosphorylation causes STATs to dissociate from the receptor, dimerize, and accumulate in the nucleus, where they regulate cytokine-responsive gene expression (120–122). The STAT family comprises 7 members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Numerous cytokines activate STAT1, STAT3, and STAT5, while STAT2, STAT4, and STAT6 are relatively specific for type I IFNs, IL-12, and IL-4, respectively (120,121). The paradigm for STAT activation is now known to be overly simplified; STATs undergo additional post-translational modifications that control their function (e.g. acetylation, serine phosphorylation), STATs can be stimulated by non-receptor kinases (e.g. Src), and STATs can operate as transcriptional regulators in the absence of tyrosine phosphorylation (120,123). Moreover, Jak2 kinase directly affects nuclear transcriptional competency by phosphorylating histone H3, which leads to dissociation of the chromatin-condenser HP1 (124). These exciting results suggest the possibility of Jaks directly regulating nuclear gene expression in pDCs and/or DCs and add a layer of complexity to cytokine-mediated transcriptional control. In the context of immune cell development, STATs have well-established roles in initiating and sustaining CD4 $^+$ T-cell lineage commitment and differentiation, B-cell generation, erythropoiesis, and granulopoiesis (125–129). Whether STATs participate in most hematopoietic lineage commitment events is less clear; however,

work from our laboratories as well as others has elucidated STAT function in pDC and DC lineage differentiation (79,130).

STAT3

STAT3 can be activated by Flt3L signaling and therefore has been considered a likely candidate to mediate pDC and DC development (130). Since complete deletion causes embryonic lethality (131), conditional ablation of *Stat3* in the hematopoietic system has been used to investigate STAT3 function in pDCs and DCs. The first report with hematopoietic STAT3-deficient mice [*Tg(Tek-cre)12Flv, Stat3^{fl/fl}*] showed a severe reduction in resident spleen CD11c⁺ DCs in steady state as well as impaired responses in lipopolysaccharide (LPS)-mediated recruitment of tissue (migratory) DCs to spleen (130). In addition, STAT3 is necessary for efficient generation of pDCs (defined by CD11c⁺CD11b⁻ surface expression) from Flt3L bone marrow cultures *in vitro* (130). In response to Flt3L treatment *in vivo*, STAT3-deficient mice fail to upregulate DC precursor and lymphoid organ DC numbers; however, CLPs and CMPs accumulate 2-3-fold relative to wildtype animals (130). Taken together, these data indicate that STAT3 controls both homeostatic amounts and Flt3L-driven production of resident DCs from pDC/DC progenitors; Flt3L responses in the progenitor compartment may be mediated by a STAT3-dependent transition from multipotent cells to DC-committed precursors. STAT3 also appears to control the amount of migratory DCs and/or their response to inflammatory signals (130).

We used *Tg(Tek-cre)12Flv, Stat3^{fl/Δ}* mice to explore the cellular and molecular mechanisms by which STAT3 controls pDC and DC generation. We found that STAT3 was required for Flt3L-dependent proliferation of lin⁻Flt3⁺ bone marrow progenitors as well as accumulation of pDCs and CD11c⁺CD11b⁺ DCs in *ex vivo* Flt3L cultures (79). Recently, we determined that pDC numbers are reduced in bone marrow and spleen of hematopoietic STAT3-deficient mice compared to wildtype control animals (Li *et al.*, unpublished results), therefore indicating that STAT3 has an obligatory role in the pDC developmental pathway in homeostatic conditions *in vivo* and in response to Flt3L signaling *in vitro*. Surprisingly, however, the low numbers of pDCs that develop from STAT3-deficient bone marrow progenitors respond to CpG-A stimulation by producing IFN- α at similar amounts compared to wildtype pDCs (79). Therefore, the data indicate that STAT3 controls Flt3L-responsive pDC/DC progenitor proliferation and resulting expansion of downstream lineages, e.g. pDCs, DCs, while it is dispensable for terminal pDC differentiation. The function of STAT3 in mediating Flt3L-responsive progenitor proliferation *in vitro* parallels the induction of MDPs by Flt3L treatment *in vivo* (72), underscoring the importance of the Flt3L-Flt3-STAT3 axis in driving expansion of the pDC/DC progenitor compartment. The role of STAT3 in MDP and CDP maintenance under steady state conditions is less clear, however, as homeostatic progenitor amounts have not been reported in hematopoietic STAT3-deficient mice. This issue is important to examine, as redundant factors could serve to maintain pDC/DC progenitor amounts in steady state conditions; identification of such factors would be of great interest in learning how to manipulate pDCs and DCs *in vitro* and *in vivo*.

In our analyses of STAT3-deficient mice, we did not find a role for STAT3 in regulating CD11c⁺CD11b⁺ DC numbers in bone marrow or spleen under homeostatic conditions (Li *et al.*, unpublished results). These results differ from the previous analysis of DC amounts in STAT3-deficient mice (130), although both studies used the *Tg(Tek-cre)12Flv* transgenic mice to direct STAT3 deletion from floxed STAT3 alleles. The observed differences suggest additional factors such as strain backgrounds or environmental conditions may affect DC maintenance in the absence of STAT3 signaling. Moreover, since Flt3 is required for the local proliferation of DC precursors in spleen (72), our data suggest Flt3 may utilize signaling cascades beyond STAT3 to control steady state DC amounts in lymphoid organs.

STAT3 is not necessary for GM-CSF-driven production of inflammatory DCs *in vitro* or GM-CSF-mediated suppression of Flt3L-dependent pDC development (79), indicating alternative pathways exist for DC and pDC regulation. However, a detailed analysis of resident spleen DC subsets as well as migratory DC amounts in STAT3-deficient animals must be undertaken to fully elucidate the action of this important transcriptional regulator *in vivo* and assess potential contributions of alternative signaling cascades in DC maintenance.

The phenotype of hematopoietic STAT3-deficient mice must be interpreted with caution and, if possible, validated by specific ablation of *Stat3* in pDC/DC progenitors. We find that conditional *Stat3* deletion is not 100% effective using the *Tg(Tek-cre)12Flv* transgene (Tie2cre), and mice with the *Tg(Tek-cre)12Flv, Stat3^{f/f}* genotype can show significant amounts of STAT3 protein in bone marrow cell lysates (Panopoulos, AD, and Watowich, SS, unpublished data). This has prompted us to analyze protein expression in all animals with the *Tg(Tek-cre)12Flv, Stat3^{f/f}* genotype and utilize only those that exhibit efficient ablation of STAT3 protein for our experiments (127). In addition, STAT3-deficient mice have numerous immune impairments and develop severe inflammatory disease with age (132), which may have indirect effects on pDC and/or DC homeostasis.

While STAT3 is typically considered to be a growth-promoting signal transducer and is, in fact, oncogenic if constitutively activated (133), STAT3 overexpression in Flt3 bone marrow progenitors instructed differentiation into pDCs and DCs *in vivo* (134). These observations suggest that STAT3 drives the expression of genes that induce pDC/DC lineage commitment. Similarly, we found that Flt3L induced the expression of key pDC transcriptional regulators (PU.1, IRF7, IRF8, SpiB) in lin⁻Flt3⁺ progenitors (79); however, it remains unclear whether Flt3L utilizes STAT3 in activating and/or sustaining the transcriptional network required for pDC development *in vivo*. Moreover, PU.1 overexpression in Flt3 bone marrow progenitors directed pDC and DC commitment, and upregulation of PU.1 as well as pDC and DC target genes was found in STAT3-overexpressing bone marrow progenitors (134). These data collectively suggest *Sfp1* (PU.1) may be a target gene of the Flt3L-Flt3-STAT3 signaling cascade. Studies from our laboratory (Yoon, D and Watowich, SS, unpublished results) as well as Dr. Pamela Hankey's group (135) implicated STAT3 in direct regulation of PU.1 in myeloid progenitors in response to G-CSF signaling or transformation with the erythroleukemia-inducing spleen focus-forming virus. STAT3 was found to interact with an upstream region of the *Sfp1* (PU.1) locus, which is located ~10kb in the 5' direction relative to the transcriptional start site, via chromatin immunoprecipitation assays (135, Yoon, D and Watowich, SS, unpublished results). These results collectively suggest *Sfp1* as a target gene for STAT3 in pDC/DC progenitors, a notion that can be examined directly by comparing PU.1 regulation in progenitors from STAT3-deficient and wildtype animals. Additional target genes with involvement in pDC/DC lineage commitment may also be regulated by STAT3. This question requires further investigation to determine the mechanism(s) of STAT3 action and could be addressed by genome-wide STAT3 ChIP-sequencing experiments and analysis of gene expression patterns in STAT-sufficient vs. STAT3-deficient MDPs or CDPs.

STAT5

The term STAT5 actually refers to two proteins, STAT5A and STAT5B, which are encoded by adjacent genes found in the vicinity of the *Stat3* gene (136). The first mouse model in which both genes were targeted for ablation was later found to be a STAT5 hypomorph (*Stat5ab^{ΔN/ΔN}*), as an N-terminally truncated STAT5 protein with partial activity was detected in certain tissues including lymphoid cells (137). The *Stat5ab^{ΔN/ΔN}* animals provide a valuable model for understanding the function of the STAT5 N-domain, e.g. revealing important role in leukemia progression (137); however, the mice cannot be considered a full deletion or null strain. Fortunately for our understanding of pDC/DC

development, studies did not begin in earnest with this hypomorphic strain as DC subsets were still being resolved when the mice were generated. Therefore timing was appropriate when the complete *Stat5a*^{-/-}*Stat5b*^{-/-} (*Stat5*^{-/-}) mice became available through Dr. Lothar Hennighausen's laboratory (138).

Using *Stat5*^{-/-} mice, we asked whether STAT5 was required for GM-CSF-mediated suppression of pDC development, GM-CSF-responsive production of inflammatory DCs *in vitro*, and DC subset generation *in vivo*. DC and pDC generation *in vivo* was determined following adoptive transfer of *Stat5*^{-/-} or *Stat5*^{+/+} fetal liver progenitors into lethally irradiated mice, as complete *Stat5* deletion causes neonatal lethality (138). These studies revealed that STAT5 is not essential for repopulation of spleen CD11c⁺CD11b⁺ DCs (79), consistent with the fact that GM-CSF is largely dispensable for DC maturation *in vivo* (58). However, CD11c⁺CD11b⁺ DC numbers were reduced in spleen, and total spleen cell counts were dramatically suppressed, in accord with the role for STAT5 in lymphoid development (139). Thus, complete reconstitution of CD11c⁺CD11b⁺ DC numbers in spleen requires STAT5, although whether DC repopulation is directly mediated by STAT5 signaling in DC precursors or depends on the lymphoid architecture of the organ remains to be determined. Analysis of resident spleen DC subsets revealed that *Stat5*^{-/-} chimeras have a higher proportion of CD8α⁺ DCs and a reduced frequency of CD4⁺CD8α⁻ DCs, suggesting STAT5 signaling regulates balanced production of these splenic DC subsets *in vivo* (79). When provided as the sole exogenous growth factor, GM-CSF is completely dependent on STAT5 to direct inflammatory DC production from lin⁻Flt3⁺ progenitors (79). Further studies are necessary to evaluate the role of STAT5 in GM-CSF-mediated inflammatory DC and migratory DC subset generation *in vivo*, and to resolve whether STAT5-activating cytokines are involved in regulating resident spleen CD8α⁺ and CD4⁺CD8α⁻ DCs.

We showed that GM-CSF and STAT5 override Flt3L signals in early stages of the pDC developmental pathway (i.e. in lin⁻Flt3⁺ progenitors) to block pDC formation (79). GM-CSF was unable to induce the conversion or maturation of terminally differentiated pDCs to DCs, confirming it operates upon a progenitor subset with pDC potential. We speculate that GM-CSF and STAT5 may act on the CDP, which expresses the GM-CSF receptor and would be found within the lin⁻Flt3⁺ bone marrow fraction (51). In agreement, pDC repopulation following bone marrow ablation was negatively regulated by STAT5 (79). Hence, the pDC/DC progenitor compartment may respond to STAT5-activating cytokines to control balanced production of the pDC and DC lineages *in vivo*. The identity of such STAT5-activating cytokines and their effect on pDC/DC subset generation and the outcome of active immune responses require further investigation.

The ability of STAT5 to inhibit pDC generation from bone marrow progenitors suggested to us that STAT5 might affect pDC/DC cell fate decisions. This idea is reminiscent of the lymphoid to myeloid lineage conversion function for GM-CSF in CLPs and early T-cell progenitors (140,141) as well as IL-2- and STAT5-mediated inhibition of Th17 development (142), which depends upon STAT3 and STAT3-activating cytokines (143,144). Furthermore, GM-CSF was recently shown to direct lineage commitment in GMPs (145). While the latter studies did not examine STAT5 function, they add to the growing body of evidence demonstrating that GM-CSF signals have instructive activity. Within the pDC/DC progenitor subset (lin⁻Flt3⁺ progenitors), we found that GM-CSF and STAT5 signaling had a broad effect on Flt3L-responsive pDC gene expression, e.g. GM-CSF-STAT5 signals suppress *Flt3*, *Tlr9*, *Irf7*, *Irf8*, and *Spib* and induce *Irf4* expression (79). To understand STAT5 suppressive mechanisms, we inspected the promoter regions of genes encoding the lineage-specific transcription factors IRF4, IRF8 and SpiB. Consensus STAT sites were found in the proximal promoter regions of *Irf8* and *Irf4*; between these two genes, we identified *Irf8* as a direct target gene of GM-CSF-activated STAT5 (79). Since IRF8 is

crucial for pDC maturation (106,107), inhibition of its expression by GM-CSF-dependent STAT5 signaling most likely enforces the blockade in pDC production. In addition, suppressed IRF8 expression may impact the expression of other regulatory transcription factors (e.g. SpiB, IRF7) (Fig. 4) and receptors (e.g. Flt3, TLR9) that are necessary for pDC development and function. It is not yet known if GM-CSF-dependent STAT5-mediated suppression of IRF8 operates in other hematopoietic lineages to regulate their development and/or function, although our preliminary studies with the D2SC/1 (DC) and J774 (macrophage) cell lines suggested this pathway may be restricted to pDC/DC progenitors (Esashi *et al.*, unpublished results). Interestingly, we found that STAT5 also negatively regulates the repopulation of splenic CD8 α^+ DCs (79), which rely on IRF8, suggesting STAT5-mediated suppression of IRF8 influences resident DC subset generation *in vivo*. Resident DC and migratory DC subsets must be more thoroughly characterized in *Stat5*^{-/-} chimeras to evaluate this possibility. In addition, the molecular pathways by which STAT5 controls pDC and DC production should be determined by analysis of STAT5 target genes in MDPs and CDPs.

STAT1

We recently found that IFN- α stimulated the generation of a pDC-related subset (IFN-pDCs) with morphologic and phenotypic similarity to classical Flt3L-generated pDCs in all aspects except TLR-triggered type I IFN production (Li *et al.*, unpublished results). STAT1 is a principal type I IFN signal transducer; therefore, we hypothesized it would be involved in mediating IFN-pDC production. This idea was supported by a recent study showing that IFN- α stimulates growth of HSCs and this occurs through a STAT1-dependent pathway (84). While IFN- α activates STAT3 and STAT1 in *lin*⁻Flt3⁺ progenitors, development of IFN-pDCs was equivalent in STAT3-deficient and wildtype bone marrow progenitor cultures (Li *et al.*, unpublished results), indicating that STAT3 is dispensable for IFN-pDC generation. By contrast, STAT1 was critical for IFN-pDC production *in vitro* and *in vivo*. Moreover, IFN-responsive STAT1 upregulates *Irf8* transcription in the DC cell line D2SC1, implicating STAT1-mediated induction of IRF8 as a mechanism contributing to production of IFN-pDCs (Li *et al.*, unpublished results). IFN- α also induced the number of *lin*⁻Flt3⁺ bone marrow progenitors, suggesting a role in pDC/DC progenitor proliferation (Li *et al.*, unpublished results). These results, taken together with studies in HSCs (84), suggest that IFN- α /STAT1 signaling may direct the expansion of the HSC population as well as enhanced commitment to the pDC/DC progenitor subset, resulting in increases in pDC/DC progenitors and IFN-pDC numbers. In addition, the data showing that IFN- α and STAT1 have hematopoietic activities (84) indicates that the use of poly (I:C) to induce conditional gene deletion with the IFN-responsive Mx-cre transgenic mouse may not be ideal for studying blood cell development.

Type I IFN-mediated suppression of DCs appears to involve a distinct pathway to control IFN-pDC generation. Specifically, inhibition of DCs by IFN signaling was shown to require STAT2 but not STAT1 (81). As STAT2 has been generally considered to operate in conjunction with STAT1 and IRF9 in type I IFN signaling (122), these data provide an important clue suggesting the potential for genes controlled by STAT2 alone. Moreover, the results indicate that separate transcriptional responses regulate IFN-dependent production of IFN-pDCs versus IFN-mediated suppression of DCs *in vivo*; however, the identity of these regulatory cascades requires continued investigation.

Conclusion and future perspectives

The available data suggest a network of cytokine-responsive STAT factors intersecting with pDC and DC lineage regulators to affect developmental decisions. Fig. 4 presents the beginning of such a model for pDCs and IFN-pDCs, along the lines of models for lymphoid

cell specification (146), in which STATs positively or negatively influence pDC/DC transcriptional regulators. In turn, these transcription factors may induce autoregulatory loops that are yet to be characterized, cross-regulate one another to instruct or sustain developmental options, and/or initiate antagonistic interactions that reject alternative cell fates (e.g. Id2-E2-2). Compared with pDCs, our understanding of resident and migratory DC lineage specification and diversification is at a more primitive stage, although we expect similar mechanisms will be utilized as those described for pDCs and other hematopoietic lineages. Future studies should be aimed at connecting extrinsic signals with cell autonomous factors, revealing epigenetic mechanisms regulating pDC and DC lineage commitment and differentiation, understanding the impact of microenvironmental signals on pDC and DC development and function, and determining how transcription factor networks affect functional outcomes (e.g. elucidation of specific gene targets regulated in differentiating pDCs and DCs). The pDC and DC lineages provide a diverse and malleable system for elucidating the regulatory events controlling hematopoiesis; in turn, this knowledge may reveal molecular targets to manipulate pDC and/or DC developmental or functional responses in clinical settings.

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Fig. 1.
Roles for cytokines in pDC and DC development.

Fig. 2.
Influence of type I IFNs on pDC and DC generation.

Fig. 3.
Lineage-restricted transcription factors and their role in pDC and DC development.

Fig. 4.
Pathways interconnecting STATs and pDC/DC lineage regulators.