# Regulation of Dendritic Cell Development by STATs

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**Abstract**
Dendritic cells (DCs) serve as a critical link between the innate and adaptive immune responses due to their ability to sense pathogens and respond by activating adaptive immune cell types. Delineating the molecular control of DC development will provide important information about the generation of natural immunity as well as approaches to regulate DCs in clinical settings. DCs are generated from hematopoietic stem cells through specialized progenitor subsets in response to cytokine and transcriptional cues, with FMS-like tyrosine kinase 3 ligand (Flt3L) and Flt3L receptor (Flt3) signaling providing a major pathway supporting homeostatic DC generation. Recent work has indicated that granulocyte-macrophage colony-stimulating factor (GM-CSF) and type I interferons (IFNs) also play important roles in regulating DC subset production. Here we review new insight into the mechanisms by which cytokine-activated STAT proteins control the DC developmental process.

## Introduction

Dendritic cells (DCs) are known as 'professional' antigen-presenting cells, unique in their capacity to maintain self-tolerance and initiate primary T- and B-cell responses. Diverse DC subsets have been reported based on phenotypic markers,

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anatomical location and functionality, with resident, migratory and plasmacytoid DCs (pDC) as the major categories (reviewed in Watowich and Liu 2010; Merad and Manz 2009). Resident and migratory DCs can be further divided into distinct classes; for example, in mouse spleen resident $CD11c^{+}$ $CD11b^{+}$ $CD4^{+}$ $CD8\alpha^{-}$ and $CD11c^{+}$ $CD11b^{-}$ $CD4^{-}$ $CD8\alpha^{+}$ DC subsets exist, generally referred as $CD8\alpha^{-}$ and $CD8\alpha^{+}$ DCs, respectively. Resident DCs are located primarily in lymphoid tissue and have also been termed conventional DCs (cDCs) while migratory subsets, which travel to lymphoid organs from peripheral sites upon activation, are additionally referred to as tissue DCs. By contrast, pDCs are found mainly in bone marrow, blood, spleen and lymph node, are defined as $CD11c^{lo}$ $CD11b^{-}$ $B220^{+}$ PDCA-$1^{+}$ $SiglecH^{+}$ plasma cell-like DCs, and are distinguished by their ability to produce abundant quantities of type I interferons (IFNs) upon viral or Toll-like receptor stimulation. In this chapter, we use the term "DC" to refer to the collective group of resident and migratory DCs, while excluding the pDC lineage, which we classify separately. Moreover, in cases where clear indications exist in the literature, we describe specific DC subsets by category such as cDC (referring to resident DCs) or tissue DC (referring to migratory DCs). It should be noted that delineation of DC subsets and molecular cues regulating their development is an intense area of investigation with continuous information emerging.

The origin of cDCs and pDCs is restricted to the lineage-negative ($lin^{-}$), $Flt3^{+}$ compartment in bone marrow (D'Amico and Wu 2003), whereas migratory DCs appear to descend from hematopoietic progenitors or monocytes (reviewed in Merad and Manz 2009). Within the $lin^{-}$ $Flt3^{+}$ progenitor subset are found macrophage-DC precursors (MDPs, $lin^{-}$ c-$kit^{hi}$ $CD115^{+}$ $CX_3CR1^{+}$ $Flt3^{+}$), which give rise to cDCs, pDCs, monocytes and macrophages upon adoptive transfer (Fogg et al. 2006; Liu et al. 2009), and a more developmentally confined progenitor, the common DC progenitor (CDP, $lin^{-}$ c-$kit^{lo}$ $CD115^{+}$ $Flt3^{+}$), which is able to generate cDCs and pDCs nearly exclusively (Naik et al. 2007; Onai et al. 2007; Liu et al. 2009). Unlike pDCs that are fully developed in the bone marrow, cDC precursors (pre-cDCs, $lin^{-}$ $CD11c^{+}$ MHC class $II^{-}$ SIRP-$\alpha^{int}$ $Flt3^{+}$) are believed to exit bone marrow and migrate to lymphoid tissues for subsequent division and terminal differentiation (Liu et al. 2009).

As with other hematopoietic lineages, the molecular regulation of DC subset specification and differentiation from hematopoietic stem cells (HSCs) is complicated and involves cellular events driven by cytokines and lineage-restricted transcription factors. Herein we focus on factors regulating cDC and pDC development, as these subsets appear to share a common progenitor and in some cases similar developmental mechanisms (Naik et al. 2007; Onai et al. 2007; Liu et al. 2009) (reviewed in Watowich and Liu 2010; Merad and Manz 2009). For instance, engagement of Flt3 with its ligand Flt3L activates the most critical signaling cascade for pDC and cDC generation in vivo, as implicated by the phenotypes of Flt3 and Flt3L gene knockout and transgenic animals (McKenna et al. 2000; Manfra et al. 2003; Waskow et al. 2008; Kingston et al. 2009). The importance of Flt3L in DC development is further demonstrated by expansion of pDC and DC populations following its administration in vivo and by its ability to induce pDC

and DC production ex vivo (Maraskovsky et al. 1996; Daro et al. 2000; O'Keeffe et al. 2002; Gilliet et al. 2002). By contrast, granulocyte/macrophage colony stimulating factor (GM-CSF) is commonly used in cell culture to generate DCs from bone marrow precursors and blood monocytes (Inaba et al. 1992), but its role in homeostatic cDC production in vivo appears to be largely dispensable or redundant with other factors, as genetic deficiency of GM-CSF or its receptor leads to only minimal reduction of cDCs (Vremec et al. 1997; Kingston et al. 2009). Moreover, GM-CSF favors DC generation yet inhibits the development of pDCs (Gilliet et al. 2002; Esashi et al. 2008). Interferon-α, a type I IFN secreted by pDCs and other blood cells upon viral infection, promotes the production of CDPs and pDCs in vivo while abrogating cDC generation (Li et al. 2011), similar to the inhibitory action of IFN-β upon cDCs (Hahm et al. 2005). Thus, Flt3L, GM-CSF and IFN-α/β have emerged as important DC regulators, albeit with distinct activities. These cytokines activate multiple signal transduction cascades, most prominently the STAT pathways. In this chapter, we focus on the mechanisms of DC development by STAT proteins.

## Flt3L-STAT3

Flt3L promotes the proliferation, differentiation and survival of many hematopoietic lineages, in addition to serving as the most critical growth factor for pDCs and cDCs in vivo and in vitro (Stirewalt and Radich 2003; Watowich and Liu 2010). Flt3L is produced by several tissues, including lymphoid organs (bone marrow, thymus and spleen), prostate, kidney, small intestine and placenta (Stirewalt and Radich 2003). The most abundant isoform of Flt3L in humans is the full-length transmembrane protein, which can be cleaved to generate a soluble isoform containing only the extracellular domain (Hannum et al. 1994; Lyman et al. 1994). Structural analysis of soluble human Flt3L revealed that it forms a noncovalently-linked homodimer with each monomer demonstrating an α-helical bundle configuration (Savvides et al. 2000). In mouse, Flt3L is present mainly as a 220 a.a. membrane-bound isoform (Lyman et al. 1995a). A soluble variant of Flt3L is also produced in humans and mice by virtue of a stop codon introduced near the end of exon 6 (Lyman and Jacobsen 1998). It is unclear whether the multiple Flt3L isoforms are functionally distinct, however all are biologically active and show no restriction in species specificity.

Flt3L signals through its receptor Flt3, also known as fetal liver kinase-2 (flk-2), stem cell kinase 1 (STK-1), or CD135. Flt3 belongs to the type III tyrosine kinase receptor family, presenting as a monomeric, membrane-bound receptor with two intracellular kinase moieties linked by a kinase-insert region (Stirewalt and Radich 2003) (Fig. 1). Flt3 is expressed primarily on early hematopoietic progenitor cells with myeloid or lymphoid potential (reviewed in Lyman and Jacobsen 1998); certain non-hematopoietic organs including placenta, gonads and brain have also been reported to express *Flt3* mRNA (Stirewalt and Radich 2003). The relatively restricted expression pattern of Flt3 within the hematopoietic system confines the

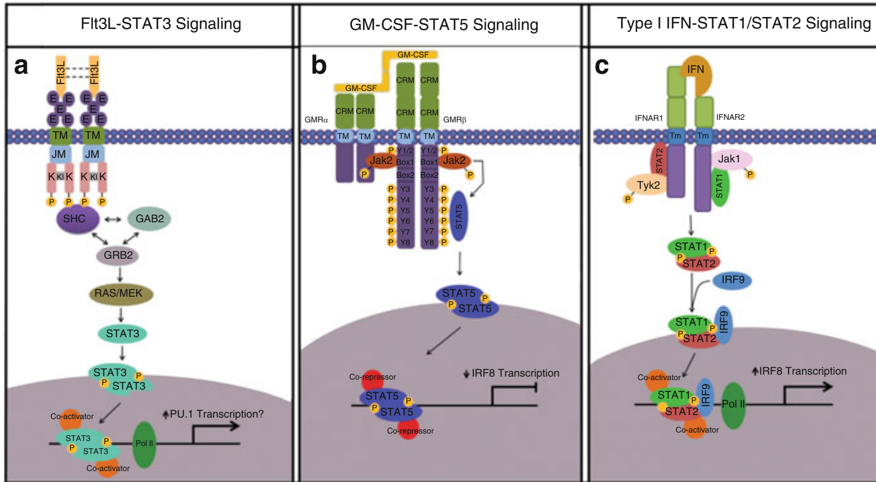


**Fig. 1** Model for cytokine-STAT signaling cascades regulating DC development. Cytokine-activated STATs have been found to regulate genes encoding critical pDC/DC-related transcription factors, signaling pathways that may influence pDC and DC development. A model of proposed signaling mechanisms is shown. STATs may also control the expression of genes controlling pDC/DC progenitor proliferation and survival as well as mature pDC and DC functions (not shown). (**a**) Flt3-STAT3. Upon Flt3L binding, Flt3 dimerizes and undergoes autophosphorylation, subsequently activating effector molecules including SHC, GAB2, GRB2 and RAS/MEK, which lead to STAT3 tyrosine phosphorylation directly or indirectly. Activated STAT3 dimerizes and translocates to the nucleus, inducing signals that mediate pDC and DC generation, potentially including direct induction of *Sfpi1* expression. Flt3 comprises five extracellular immunoglobulin-like domains (E), a transmembrane domain (TM), a juxtamembrane domain (JM) and two tyrosine-kinase domains (K) linked via a kinase insert region (KI). (**b**) GM-CSF-STAT5. GM-CSF stimulates the formation of hexameric (2GMRα:2βc:2GM-CSF) or dodecameric (2x hexameric) ligand:receptor complexes, which activate JAK2 and, subsequently, STAT5. STAT5 dimers translocate to the nucleus where they suppress *Irf8* gene expression, inhibiting pDC development. GMRα contains a CRM domain, a TM domain and a cytoplasmic domain while βc consists of two CRMs, a TM domain and a cytoplasmic domain that associates with Jak2. (**c**) Type I IFN-STAT1/2. Type I IFN stimulation activates Tyk2 and Jak1, which are associated with IFNAR1 and IFNAR2, respectively. Jak activation leads to phosphorylation of STAT2 and STAT1, which may induce STAT1 homodimerization as well as STAT2 and STAT1 interaction with IRF9 to form STAT1:STAT2:IRF9 complexes (known as ISGF3). ISGF3 and/or STAT1 homodimers (not shown) accumulate in the nucleus and induce *Irf8* expression, potentially enhancing pathways that stimulate/support pDC differentiation. The signaling network involved in type I IFN-mediated DC repression is unclear

action of Flt3L, which is essential for normal hematopoiesis and immune system function. Low amounts of circulating Flt3L (Lyman et al. 1995b) and the proximity of Flt3L-producing cells suggest that paracrine stimulation of $Flt3^{+}$ hematopoietic progenitors may occur in the bone marrow microenvironment; autocrine mechanisms have also been proposed, however this remains to be established (Stirewalt and Radich 2003).

Flt3L binding leads to dimerization of Flt3 and concomitant juxtaposition of the cytoplasmic tyrosine kinase domains, inducing rapid autophosphorylation (within minutes) of the receptor (Turner et al. 1996). The activated Flt3L-Flt3 complex, which is rapidly internalized (Turner et al. 1996), stimulates multiple signaling cascades that seem to be tissue and cell type-specific (Dosil et al. 1993; Zhang et al. 1999). In the Flt3$^{+}$ Ba/F3 cell line, activated Flt3 stimulates tyrosine phosphorylation of SH2-containing sequence protein (SHC), the inositol phosphatase SHIP, the protein phosphatase SHP2 and GRB2-binding protein (GAB2), which subsequently initiates multiple signal transduction cascades including the phosphatidylinositol 3-kinase (PI3K)/Akt and RAS/mitogen-activated protein kinase (MAPK) pathways (Marchetto et al. 1999; Zhang and Broxmeyer 2000). STATs are thought to be downstream intermediates following activation of RAS, RAF or MAPK/ERK kinases (MEK), without involvement of JAK activity (Zhang et al. 2000) (Fig. 1). By contrast, in circumstances in which Flt3 is overexpressed or constitutively activated, STAT5 is stimulated through interaction with phosphorylated tyrosines 589 and 591 within the Flt3 juxtamembrane region (Spiekermann et al. 2003; Rocnik et al. 2006). Phosphorylation of these tyrosine residues, however, does not occur upon physiological Flt3L stimulation (Rocnik et al. 2006), and STATs may be activated directly via recruitment to the receptor or indirectly through Src kinases (Heiss et al. 2006). In lin$^{-}$ Flt3$^{+}$ pDC/DC progenitors, STAT3 is the major STAT activated, becoming tyrosine phosphorylated within minutes of Flt3L addition (Esashi et al. 2008), suggesting direct stimulation via Flt3 (Fig. 1).

Flt3L was first described as an effective DC growth factor in mice receiving daily injection of the recombinant protein, a regimen that stimulates a significant increase in pDCs and DC subsets in bone marrow, blood, lymphoid organs and other tissues (e.g. thymus, spleen, gastro-intestinal lymphoid tissue and liver) (Maraskovsky et al. 1996). Subsequently, Flt3L was found to significantly boost DC generation from human blood ex vivo in the presence of GM-CSF and IL-4 (Hubert et al. 1998), or to promote pDC and DC production in human and mouse bone marrow cultures (Blom et al. 2000; Gilliet et al. 2002). Inducible expression of Flt3L in vivo also results in massive expansion of pDCs and DCs in multiple organs (Manfra et al. 2003). DCs that develop in response to Flt3L in vivo and ex vivo appear to correspond to pDC and DC populations that are generated in homeostatic conditions in vivo (Naik 2008). Importantly, Flt3L treatment also stimulates the development and/or accumulation of MDPs and CDPs from HSCs (Waskow et al. 2008) (Li and Watowich, unpublished data), highlighting its critical function throughout the DC developmental process. Correspondingly, ablation of Flt3L-Flt3 signaling by genetic disruption (McKenna et al. 2000; Waskow et al. 2008; Kingston et al. 2009) or treatment with Flt3 inhibitors (Tussiwand et al. 2005) leads to severe reduction (>90%) in pDC and DC amounts, and a mild decrease (30–40%) of bone marrow MDPs (Kingston et al. 2009). By contrast, enforced expression of Flt3 in Flt3$^{-}$ hematopoietic progenitors, which normally lack DC potential, enables their development into pDCs and DCs (Onai et al. 2006), suggesting Flt3 signaling may have instructive function in DC lineage development.

In a first attempt to explore the molecular mechanisms involved in Flt3L-induced DC development, Laouar et al. reported that conditional deletion of STAT3 in the bone marrow caused a profound loss of splenic CD11c$^+$ cells in C57BL/6 mice and impaired the generation of both pDC and DC subsets from Flt3L-supplemented bone marrow cultures ex vivo (Laouar et al. 2003). In addition, hematopoietic STAT3 deficiency abrogated Flt3L-stimulated induction of CD11c$^+$ DCs as well as common lymphoid progenitors (CLPs), which can serve as DC precursors, yet had no effect on HSCs or common myeloid progenitors (CMPs) (Laouar et al. 2003). These data suggested a role for STAT3 in homeostatic maintenance/development of DCs as well as Flt3L-driven generation of DCs, pDCs and their progenitors. Subsequently, our laboratory demonstrated that STAT3 is essential for the proliferation of lin$^-$ Flt3$^+$ bone marrow progenitors in response to Flt3L (Esashi et al. 2008), consistent with its critical role in Flt3L-dependent generation of pDCs and DCs in vitro (Laouar et al. 2003; Esashi et al. 2008). Moreover, we found that STAT3-deficient pDCs retain their ability to produce type I IFNs upon TLR9 stimulation (Esashi et al. 2008). Collectively, these results suggest that STAT3 mediates growth-promoting signals in pDC/DC progenitors elicited by Flt3L stimulation, yet is dispensable for terminal pDC differentiation. Under steady state conditions, however, pDC/DC progenitors are found at similar amounts in hematopoietic STAT3-deficient mice relative to littermate controls (Li et al. 2011), indicating STAT3 is not required for homeostatic maintenance of the progenitor compartment. Furthermore, recent data indicates that STAT3 deletion in the hematopoietic system (Li et al. 2011) or in CD11c$^+$ cells (Melillo et al. 2010) leads to a significant reduction in pDCs in bone marrow and spleen while CD11c$^+$ CD11b$^+$ cDCs are present at near normal numbers and proportions; these results agree with the concept that STAT3 is necessary for proliferation of pDC progenitors (Esashi et al. 2008) yet indicate that cDCs or their precursors may be regulated by STAT3-independent mechanisms under homeostatic conditions in vivo. Flt3 controls cDC proliferation/survival in spleen in steady state (Waskow et al. 2008), thus the fact that CD11c$^+$ CD11b$^+$ cDCs remain at normal amounts in STAT3-deficient mice suggests that Flt3-driven cDC proliferation in the periphery employs signal cascades independent of STAT3. The reason for the discrepancy in the role for STAT3 in DC development between different reports (Laouar et al. 2003; Melillo et al. 2010) remains unclear, however the recent work underscores the critical role for STAT3 in pDC homeostasis in vivo (Melillo et al. 2010) (Li et al. 2011). Significantly, ectopic expression of STAT3 in Flt3$^-$ progenitors not only initiates pDC development from this subset, which is normally unable to generate pDCs, but also upregulates Flt3 expression (Onai et al. 2006). These results suggest that STAT3 may mediate instructive signals for pDC lineage development and potentially reinforce this signaling network via Flt3 induction.

Flt3L stimulates the expression of several DC-related transcription factors, including *Sfpi1* (encoding PU.1), *Ifr8* and *Spib* (Esashi et al. 2008). An important question centers on whether STATs serve a role in activating and/or maintaining the DC lineage-specific transcriptional network. STAT3 has been shown to regulate

*Sfpi1* expression by binding the distal promoter region in primary erythroid progenitors or myeloid tissue culture cells (Hegde et al. 2009; Yoon and Watowich, unpublished data). These results, together with the observation that *Sfpi1* mRNA is induced by STAT3 overexpression in $Flt3^{-}$ bone marrow progenitors (Onai et al. 2006), suggest that *Sfpi1* may be a direct target of the Flt3-STAT3 signaling cascade in pDC/DC progenitors/precursors (Fig. 1). Recently, PU.1 was shown to induce Flt3 expression in pDC/DC progenitors in a dose-dependent manner (Carotta et al. 2010), suggesting the presence of a self-regulatory loop of Flt3 transcription mediated by STAT3 and PU.1 following Flt3 signaling.

## GM-CSF-STAT5

GM-CSF is the most commonly used growth factor in the laboratory and clinic to generate DCs from peripheral blood and/or bone marrow. GM-CSF is normally undetectable in circulation, but is readily induced in many cell types in response to multiple stimuli or disease conditions (Hamilton and Anderson 2004). As revealed by X-ray crystallography, human GM-CSF is characterized by a highly compact four α-helix bundle structure containing a hydrophobic core (Rozwarski et al. 1996). Human and mouse GM-CSF display a high degree of homology in sequence and predicted structure, yet there is little species cross-reactivity due to the fact that distinct residues are required for receptor binding (Shanafelt et al. 1991).

GM-CSF receptor (GMR) is widely expressed on hematopoietic cells at low density (100–1,000 receptors/cell) (Guthridge et al. 1998). The heteromeric receptor is composed of a cytokine-specific α chain (GMRα) and a common β (βc) chain that is shared with receptors for IL-3 and IL-5 (Gearing et al. 1989; Hayashida et al. 1990; Kitamura et al. 1991). Both GMRα and βc chains belong to the class I cytokine receptor superfamily, and are characterized by the presence of the cytokine-receptor homology module (CRM) in the extracellular domain, which contains four conserved cysteine residues, a Trp-Ser-X-Trp-Ser motif (WSXWS motif) and tandem fibronectin type III domains (Bazan 1990). An "elbow" region formed by a fold between the fibronectin type III domains in GMRα serves as the critical ligand binding interface (Hansen et al. 2008), similar to growth hormone binding to the growth hormone receptor extracellular region (de Vos et al. 1992). The GMRα consists of one CRM and binds to GM-CSF at low affinity (Gearing et al. 1989). By contrast, the βc chain contains 2 CRMs and was reported to present as a membrane-bound homodimer in the absence of ligand stimulation (Fig. 1) (Hayashida et al. 1990; Murphy and Young 2006). While βc is the principal signaling component in cells expressing GM-CSF, IL-3 or IL-5 receptors, and is required for high affinity ligand binding, it is clear that different α subunits are required for cytokine-specific signal transduction (Hayashida et al. 1990; Kitamura et al. 1991; Tavernier et al. 1991; Geijsen et al. 2001). Recently, a hexameric complex consisting of 2 βc: 2 GMRα: 2 GM-CSF was identified by crystal structure analysis; this structure may further assemble into a higher order dodecamer (12-mer) (Hansen et al. 2008). The dodecameric ligand-receptor complex provides a structural basis for clustering the signaling βc subunits, and enables

association of GMRα cytoplasmic domains, which may also participate in signal transduction (Lia et al. 1996).

The βc chain constitutively associates with the tyrosine kinase Jak2, which is critical for GMR signal transduction (Parganas et al. 1998), via a conserved Box 1 motif in the membrane-proximal region of the cytoplasmic domain (Quelle et al. 1994). Jak2 clustering upon ligand binding activates the kinase by a trans-phosphorylation mechanism; activated Jak2 subsequently phosphorylates tyrosine residues in the intracellular region of the GMR. Early studies with Ba/F3 cells expressing a dominant negative Jak2 isoform demonstrated that mutant Jak2 suppressed phosphorylation of βc and inhibited GM-CSF-induced activation of immediate response genes (Watanabe et al. 1996), indicating the importance of Jak2 for GM-CSF signaling. This was confirmed by studies with fetal liver progenitors from *Jak2*$^{-/-}$ mice, which failed to respond to GM-CSF and IL-3 (Parganas et al. 1998), demonstrating a nonredundant role for Jak2 in response to cytokine signals employing βc. Activated Jak2 and βc recruit SH2 and PTB domain-containing proteins to the GMR complex and initiate multiple signaling cascades, including STAT, Ras/MAPK and PI3K/Atk pathways (Hercus et al. 2009). STAT5A and STAT5B are the predominant STATs activated by GM-CSF. Sakurai et al. have indicated that each of the eight phosphorylated tyrosine residues in βc can serve as a docking site for STAT5 in a GM-CSF-dependent in vitro system (Sakurai et al. 2000), although it is unclear whether all βc tyrosines are functionally similar in vivo. Studies in Ba/F3 cells show that Y577 in βc mediates GM-CSF-dependent phosphorylation of SHC, while Y577, Y612, or Y695 appear to be similar in their ability to induce phosphorylation of SHP2 (Okuda et al. 1997). βc also associates with other tyrosine kinases, such as Lyn, Btk and Fyn, although the function of these interactions remain largely unknown (Geijsen et al. 2001).

GM-CSF is a potent growth factor for DCs, however it has a striking suppressive activity on pDC generation in cultures with total bone marrow or purified progenitor cells, indicating its potential to block pDCs at an early developmental stage (Gilliet et al. 2002; Esashi et al. 2008). Elevated circulating amounts of GM-CSF in mice carrying a GM-CSF-encoding transgene (Vremec et al. 1997) or in animals treated with recombinant GM-CSF (Daro et al. 2000; O'Keeffe et al. 2002) leads to increases in both CD8$\alpha^+$ and CD8$\alpha^-$ cDC numbers in lymphoid organs, demonstrating that GM-CSF can drive cDC generation in vivo. By contrast, mice carrying targeted null mutations of the GM-CSF or βc genes show marginal reduction in cDC populations in thymus, spleen and lymph node (Vremec et al. 1997; Kingston et al. 2009). βc$^{-/-}$ mice lack responsiveness to GM-CSF and IL-5 yet retain the ability to respond to IL-3, due to the presence of an alternative murine IL-3 receptor (Hara and Miyajima 1992; Nicola et al. 1996). Taken together, these results indicate that GM-CSF has a limited role in cDC homeostasis, and this may be due to the presence of compensatory cytokine signals including IL-3 and Flt3L. However, during conditions in which GM-CSF is expressed at elevated amounts, such as inflammation or infection, GM-CSF may boost the production of DCs from pDC/DC progenitors and/or monocytes. The DCs that arise in these conditions do not appear to correspond to DCs present in steady state in mouse and are considered

to be a distinct "inflammatory" population (reviewed in Naik 2008). In fact, evidence for a monocyte to DC transition in vivo during inflammatory conditions has been reported. For example, $CD11c^{+}$ DCs differentiated from adoptively-transferred monocytes can be recovered from the spleens of recipient mice with systemic inflammation (Naik et al. 2006) or from inflamed peritoneum induced by thioglycollate treatment (Geissmann et al. 2003). Moreover, a novel inflammatory DC population secreting high levels of TNF-α and iNOS (Tip DCs) was reported during *L. moncytogenes* infection (Serbina et al. 2003), which is accompanied by elevated cytokine production. Hence, a principal role for GM-CSF may be to enhance the amount of antigen-presenting cells during inflammation or infection by stimulating production of inflammatory DCs.

The numerous signaling pathways elicited by GM-CSF posed challenges for specifying the roles of individual factors. As STAT5 is strongly activated in pDC/DC progenitors stimulated with GM-CSF, our group determined whether STAT5 is involved in regulating DC development. We found that STAT5 is essential for the suppressive activity of GM-CSF on pDC generation in vitro (Esashi et al. 2008). Moreover, bone marrow chimeric *Stat5a*$^{-/-}$ *Stat5b*$^{-/-}$ mice (termed here *Stat5*$^{-/-}$) demonstrate increased pDC proportions and decreased cDC frequencies in bone marrow and spleen compared to *Stat5*$^{+/+}$ chimeras, suggesting that STAT5 inhibits homeostatic pDC development in vivo (Esashi et al. 2008). Further analysis within the $CD11c^{+}$ splenic population showed an increase in the $CD11b^{-}$ $CD8\alpha^{+}$ subset proportion and a reduction in the $CD11b^{+}$ $CD4^{+}$ frequency, suggesting STAT5 signaling may also influence cDC differentiation in the spleen. By contrast, STAT5 is not required for Flt3L-driven pDC or DC development from $lin^{-}$ $Flt3^{+}$ bone marrow progenitors (Esashi et al. 2008). GM-CSF is believed to exert its suppressive function on pDC development at a pDC/DC progenitor stage since it blocks pDC generation from the $lin^{-}$ $Flt3^{+}$ subset, while terminally differentiated pDCs do not convert or develop into DCs in response to GM-CSF. In addition, GM-CSF alone is able to stimulate pDC production from *Stat5*$^{-/-}$ $lin^{-}$ $Flt3^{+}$ progenitors suggesting that GM-CSF can induce pDC development in the absence of STAT5 (Esashi et al. 2008). Consistent with our ex vivo results, GM-CSF delivery by hydrodynamic gene transfer markedly inhibits pDC generation in vivo, and this inhibition is partially abrogated in hematopoietic STAT5-deficient mice [i.e. *Tg (Tek-cre)12Flv, Stat5*$^{f/f}$] (Li et al. 2011) (Li and Watowich, unpublished data). Taken together, these results suggest that STAT5 functions as a negative signal for pDC development in steady state conditions as well as "emergency" situations with high circulating amounts of GM-CSF.

To explore how GM-CSF employs STAT5 to exert its suppressive activity on pDCs, the expression of DC-related transcription factors was compared in *Stat5*$^{-/-}$ and *Stat5*$^{+/+}$ $lin^{-}$ $Flt3^{+}$ progenitors stimulated with Flt3L or Flt3L+GM-CSF. As reported (Esashi et al. 2008), GM-CSF inhibited the expression of *Irf7*, *Irf8* and *Spib* induced by Flt3L, but promoted *Irf4* expression, in accordance with the function of these transcription factors in pDC versus cDC production (i.e. IRF7, IRF8 and SpiB are important for pDC development while IFR4 is required for $CD11c^{+}$ $CD8\alpha^{-}$ cDCs). Inspection of proximal promoter regions revealed

consensus STAT sites in the *Irf4* and *Irf8* genes, and further analysis indicated that *Irf8* transcription is directly inhibited by STAT5 upon GM-CSF stimulation (Esashi et al. 2008). IRF8 is essential for pDC development and maturation, and deletion of *Irf8* leads to a profound loss of pDCs and CD8$\alpha^+$ cDCs (Schiavoni et al. 2002). Hence, the significant suppression of *Irf8* expression by GM-CSF-STAT5 signaling is likely to contribute to GM-CSF-mediated inhibition of pDC development (Esashi et al. 2008) (Fig. 1).

## Type I IFN-STAT1/STAT2

Type I IFNs comprise a large family consisting of multiple IFN-α subtypes, a single IFN-β and other members such as IFN-κ, IFN-ε and IFN-ω. The IFNs exhibit a wide spectrum of activities in the immune system, including anti-viral effects, pro-apoptotic activity and APC-stimulating capability (Stark et al. 1998; Pestka 2000; Biron 2001; Theofilopoulos et al. 2005; Trinchieri 2010). While pDCs are considered to be a primary source of type I IFN secretion upon viral infection (Siegal et al. 1999), many other cells types, including leukocytes, T cells and NK cells, produce type I IFN upon activation. All type I IFNs bind and signal through a single receptor composed of two subunits, IFNAR1 and IFNAR2, which is expressed by many cell types (reviewed in Stark et al. 1998; Theofilopoulos et al. 2005). IFNAR1 exists primarily as a single isoform, but alternative processing of IFNAR2 transcripts produces multiple isoforms that possess identical IFN-binding sequences in the extracellular domain (Domanski et al. 1995; Lutfalla et al. 1995). Moreover, the two subunits have distinct three-dimensional structures as the extracellular domain of IFNAR1 contains two putative cytokine binding sites formed by four fibronectin type III repeats, while IFNAR2 contains a single putative cytokine binding site (Fig. 1) (Uze et al. 1995). Interaction of type I IFNs is proposed to involve two-step process whereby ligand engages IFNAR2 initially and subsequently IFNAR1 is recruited to the complex (reviewed in Stark et al. 1998; Theofilopoulos et al. 2005). Full-length IFNAR2 is required for activation of JAK-STAT signal transduction (Lutfalla et al. 1995).

Like other cytokine receptors, IFNAR1 and IFNAR2 lack intrinsic enzymatic activity, however their cytoplasmic domains noncovalently associate with the JAK kinases Tyk2 and Jak1, respectively (Colamonici et al. 1994a, b; Gauzzi et al. 1996). In vitro binding assays revealed a minimum sequence in IFNAR1, comprising residues 479–511, as the Tyk2 interaction site; this region is proximal to the inducible STAT2 docking site at Y466. By contrast, the Jak1 binding region in IFNAR2 was localized to residues 300–346, which are nearby the constitutive STAT2-docking region at residues 404–462 (Mogensen et al. 1999). In addition, IFNAR2 also binds constitutively to STAT1 in the presence of STAT2 (Li et al. 1997). Engagement of IFNAR by ligand results in phosphorylation of Jak1, Tyk2 and both receptor subunits. Subsequently, STAT2 interacts with phosphorylated Y466 in IFNAR1 via its SH2 domain. This is thought to position STAT2 and STAT1 in the correct configuration to become tyrosine phosphorylated, leading to

their heterodimerization and dissociation from the receptor complex (reviewed in Stark et al. 1998). STAT1:STAT2 heterodimers bind to p48/IRF9 to form the multimeric protein complex ISGF3, which stimulates transcription of type I IFN genes (Stark et al. 1998; Platanias 2005). STAT1 homodimers may also form in response to IFNAR activation (Platanias 2005). Of note, IFNAR1 was reported to bind to STAT3 at residues 525–544 (Yang et al. 1996), however the involvement of STAT3 in the type I IFN signaling pathway is unclear. In addition to the JAK-STAT pathway, type I IFNs can activate other signaling cascades including the MAPK cascade and PI3K (Platanias 2005).

Many investigators have reported that type I IFNs promote terminal differentiation and maturation of DCs by stimulating expression of MHC and costimulatory molecules (Trinchieri 2010). More recently, it was shown that IFN-α stimulates HSCs to exit quiescence and to enter the cell cycle via a STAT1-dependent mechanism (Essers et al. 2009); these results highlight the potential for IFN-α to induce the development of hematopoietic lineages. Consistent with this, we found that IFN-α hydrodynamic gene transfer (IFN-α HGT), which elicits elevated IFN-α amounts in the blood similar to viral infection (e.g. 2–3 ng/mL), induced an approximate twofold increase in bone marrow pDC/DC progenitor numbers (i.e. $lin^{-}$ $Flt3^{+}$ cells) after 4 days of treatment (Li et al. 2011). In addition, IFN-α stimulated pDC production from pDC/DC progenitors in vitro in the presence of a primary DC growth factor, such as Flt3L or GM-CSF. Thus, the enhanced production of pDCs in bone marrow and spleen of mice that received IFN-α HGT suggests two developmental processes: the generation of pDC/DC progenitors from HSC and the differentiation of pDCs from pDC/DC progenitors. On the other hand, high dose IFN-β treatment was shown to suppress development of MHC class $II^{+}$ $CD11c^{+}$ DCs in vivo and in vitro (Hahm et al. 2005). The suppression of DCs was also observed during infection with measles virus and LCMV (Hahm et al. 2005), while enhanced pDC production was found following vesicular stomatitis virus infection (Li and Watowich, unpublished data). Zuniga reported that LCMV infection drives differentiation of bone marrow pDCs to DCs in the presence of Flt3L by upregulating CD11b and suppressing B220 expression via IFNAR-dependent mechanisms (Zuniga et al. 2004), however IFN-α signaling does not appear to stimulate conversion between pDCs and DCs or vice versa (Li et al. 2011). Thus, type I IFNs exert multiple effects on the DC developmental pathway and may distinctly regulate pDC generation and DC suppression.

We found that pDCs that were generated in the presence of IFN-α (IFN-α-conditioned pDCs) share many features with pDCs that develop in response to Flt3L, however the IFN-α-conditioned pDCs fail to secrete type I IFN upon TLR7 and TLR9 stimulation, but produce enhanced amounts of the proinflammatory cytokines IL-6, IL-23 and TNFα. Furthermore, IFN-α-conditioned pDCs preferentially facilitate the differentiation of Th17 cells compared to pDCs that develop in steady state or in response to Flt3L (Li et al. 2011). Genetic deletion of STAT1 abrogates the development of pDCs elicited by IFN-α in vitro or IFN-α HGT in vivo, indicating an indispensable role for STAT1 in their production (Li et al. 2011). *Stat1*$^{-/-}$ mice display normal amounts of pDCs and DCs in bone marrow

and spleen (Li et al. 2011), indicating STAT1 is not necessary for DC homeostasis in these organs. Strikingly, however, *Stat1*$^{-/-}$ mice are deficient in Peyer's patch pDCs, a DC population that closely resembles IFN-α-conditioned pDCs in terms of phenotypic markers, transcription factor expression profiles and functional features (i.e. lack of IFN secretion) (Contractor et al. 2007; Li et al. 2011). This finding suggests that IFN-STAT1 signals may play important roles in pDC development in the gut and under inflammatory conditions where type I IFNs are produced. By contrast, DC suppression induced by viruses or IFN-β was dependent on STAT2, but independent of STAT1 (Hahm et al. 2005). Given the fact that IFNAR2 associates with STAT1 in a STAT2-dependent manner (Li et al. 1997) and IFN-β binding to IFNAR2 alone can stimulate early transcription of IFN-responsive genes (Lewerenz et al. 1998), it is possible that STAT2-mediated initial transcripts are sufficient to block DC development in response to IFN-β. No alteration in DC subsets has been reported in *Stat2*$^{-/-}$ animals (Hahm et al. 2005), suggesting STAT2 is not essential for DC homeostasis.

Analysis of the expression of DC-specific transcription factors in response to IFN-α suggested that IFN-α induced *Irf8* transcription in pDC/DC progenitors by a mechanism involving direct interaction of IFN-α-activated STAT1 with the *Irf8* promoter (Li et al. 2011). Since IRF8 is important for pDC development, this signaling cascade may account, at least in part, for increased pDC production in response to IFN-α (Fig. 1) (Li et al. 2011). IFN-α was also reported to enhance *IRF8* expression in human NK and T cell cultures (Lehtonen et al. 2003), as well as in patients with chronic myelogenous leukemia (Schmidt et al. 1998), but the underlying mechanisms have remained unresolved until recently. In terms of DC development, virus-induced type I IFNs do not seem to enhance DC apoptosis, but rather inhibit cell proliferation to limit DC expansion (Hahm et al. 2005). This is consistent with the anti-proliferative role for IFN-α and IFN-β (reviewed in Stark et al. 1998). Detailed analysis of how type I IFN-responsive STAT1 and/or STAT2 regulate the cell cycle machinery in DCs will extend our understanding of type I IFN-mediated DC suppression. In addition, IFN-α was suggested to inhibit IL-2-stimulated STAT5 DNA binding in T cells (Erickson et al. 2002); GM-CSF, on the other hand, blocks IFN-α-induced STAT1 activation (Kasper et al. 2007). This cross-inhibition by STAT1 and STAT5 signals are in accordance with their unique roles in pDC versus DC development, and may factor into their disparate activities in these lineages.

## Concluding Remarks and Future Perspectives

Flt3L, GM-CSF and type I IFNs are three well-studied cytokines that signal through their cognate receptors to regulate pDC and DC lineage commitment and differentiation. In each system, STATs are activated and interact with the proximal promoter regions of certain pDC- and DC-specific transcription factor genes (Fig. 1). These signals may instruct and/or reinforce the developmental decision of pDC/DC progenitors. A major focus for the future is uncovering genome-wide STAT targets in pDCs, DCs and their progenitors, and determining how STAT-regulated genes

participate in pDC/DC lineage development. In addition, other cytokines, such as thrombopoietin, M-CSF and IL-3, can independently regulate DC generation, or cooperate with other DC growth factors to influence DC development, yet the intracellular signaling pathways they employ remain unknown. The interplay and cross-regulation among multiple signaling pathways and numerous transcription factors are complex, and mechanisms by which these events regulate DCs remain as a challenging question. Compared to pDCs, less is known about the signaling networks involved in cDC subset (e.g. CD8$\alpha^{+}$ vs. CD8$\alpha^{-}$ cDC) diversification, an area that should be explored in future studies. Unraveling these mechanisms is important for learning how to manipulate pDC and cDC amounts, as well as their effects on innate and adaptive immune functions, and may aid in our ability to target pDC and DC lineages in clinical therapies for cancer or immune disease.

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