Regulation of dendritic cell development by GM-CSF: molecular control and implications for immune homeostasis and therapy

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Dendritic cells (DCs) represent a small and heterogeneous fraction of the hematopoietic system, specialized in antigen capture, processing, and presentation. The different DC subsets act as sentinels throughout the body and perform a key role in the induction of immunogenic as well as tolerogenic immune responses. Because of their limited lifespan, continuous replenishment of DC is required. Whereas the importance of GM-CSF in regulating DC homeostasis has long been underestimated, this cytokine is currently considered a critical factor for DC development under both steady-state and inflammatory conditions. Regulation of cellular actions by GM-CSF depends on the activation of intracellular signaling modules, including JAK/STAT, MAPK, PI3K, and canonical NF-κB. By directing the activity of transcription factors and other cellular effector proteins, these pathways influence differentiation, survival and/or proliferation of uncommitted hematopoietic progenitors, and DC subset–specific precursors, thereby contributing to specific aspects of DC subset development. The specific intracellular events resulting from GM-CSF–induced signaling provide a molecular explanation for GM-CSF–dependent subset distribution as well as clues to the specific characteristics and functions of GM-CSF–differentiated DCs compared with DCs generated by fms-related tyrosine kinase 3 ligand. This knowledge can be used to identify therapeutic targets to improve GM-CSF–dependent DC-based strategies to regulate immunity. (*Blood*. 2012;119(15):3383-3393)

The role of GM-CSF in the regulation of DC homeostasis

Dendritic cells (DCs) constitute a crucial and heterogeneous fraction of the hematopoietic system, with an essential role in the induction and regulation of immunity.[1] Because DCs are relatively short-lived, they are continuously replenished from bone marrow-, blood-, or tissue-derived precursors that are different for the distinct DC subsets.[2] The ontogeny of DC subsets belonging to one of the 3 main categories defined elsewhere,[2,3] migratory DCs, lymphoid-resident DCs, and plasmacytoid DCs, is shown in Figure 1. Supplemental Tables 1 and 2 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) show the phenotype and assigned classification of different DC populations that have been identified in distinct tissues in vivo or can be generated through specific culture methods. Efficient DC development from hematopoietic stem cells (HSCs) involves proliferation and survival as well as phenotypic and functional differentiation of progenitors with gradually restricted developmental options.

GM-CSF was the first cytokine shown to efficiently promote DC development in vitro[4] and has been used to induce DC differentiation from human monocytes[5] as well as human and mouse hematopoietic progenitor cells.[6-8] Significantly increased DC numbers have been found in the spleen and thymus of mice injected with GM-CSF or transgenic mice overexpressing GM-CSF,[9,10] suggesting that GM-CSF can promote DC expansion in vivo. However, only a marginal reduction in lymphoid organ DCs was found in mice lacking GM-CSF or the GM-CSF receptor (GM-CSFR).[10] In later studies, mice lacking GM-CSF or the GM-CSFR were shown to have substantially reduced numbers of migratory DCs in skin and gut,[11-13] indicating that development of these subsets requires GM-CSF under steady-state conditions. The 3-fold decrease in lymph node DCs observed earlier[10] probably reflects the loss of these migratory DCs, without alterations in the lymphoid-resident DC subset. Besides subset-specific GM-CSF dependency, GM-CSF can also negatively affect the development of specific DC subsets, as demonstrated for plasmacytoid DCs[14] (Figure 1). Interestingly, although GM-CSF inhibits commitment of bone marrow progenitors to the plasmacytoid DC lineage,[14] it supports the survival and can even initiate terminal differentiation of circulating interferon-producing cells, the direct precursors of plasmacytoid DCs.[15] Because the secretion of GM-CSF, which circulates at very low concentrations under steady-state conditions, is increased during infection and inflammation,[16] its influence on the composition of the DC pool becomes more pronounced during inflammation. As an illustration, inflammation-driven monocyte conversion to murine splenic DCs[3,17] and DCs generated during acute inflammatory arthritis or antigen-induced peritonitis[18] was reported to depend on GM-CSF. Furthermore, besides skewing the development of specific DC subsets, GM-CSF induces development of DCs with a relatively immunogenic phenotype and functionality, which resemble TNF-α and inducible nitric-oxide synthase-producing DCs[19] that develop during infection.[3]

Thus, the role of GM-CSF in DC development appears situation- as well as subset-specific. Under steady-state conditions, GM-CSF supports migratory DC development, whereas its effect on other subtypes is either redundant or even detrimental (Figure 1). Increased GM-CSF production during the onset of inflammation contributes to rapid differentiation of precursor DCs (pre-DCs)

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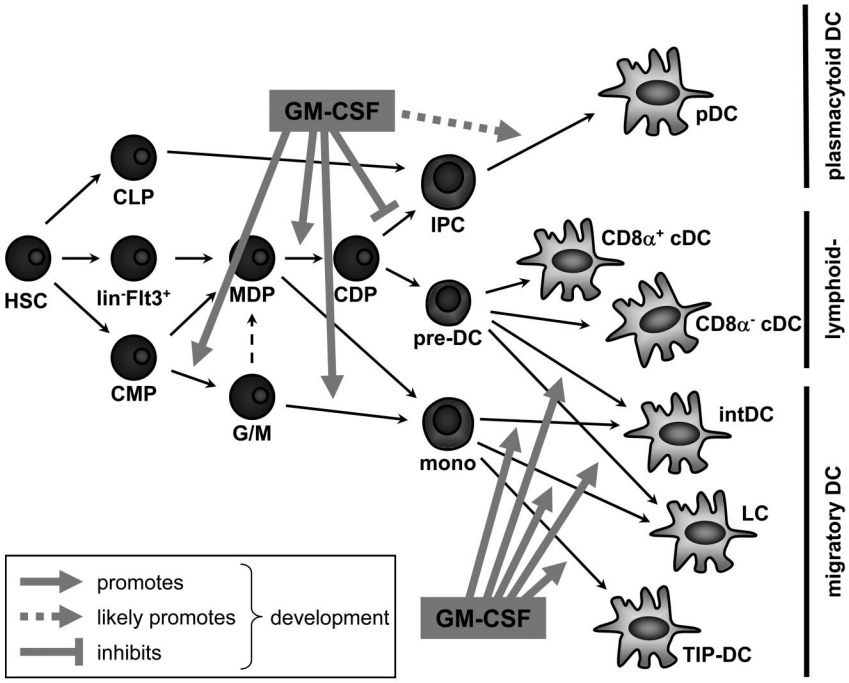

Figure 1. The role of GM-CSF during DC subset development. DCs are derived from HSCs through gradually restricted precursors. DC subsets can be classified into 3 main categories: migratory DCs, lymphoid-resident DCs, and plasmacytoid DCs. Supplemental Tables 1 and 2 show the phenotype and assigned classification of different DC populations that have been identified in distinct tissues in vivo or can be generated through specific culture methods. GM-CSF supports development of the common DC progenitor (CDP) and of the granulocyte/macrophage progenitor (G/M). Commitment of the CDP toward the plasmacytoid DC lineage is inhibited by GM-CSF, but terminal differentiation of committed plasmacytoid DC precursors (interferon-producing cells [IPCs]) is probably supported by GM-CSF. In contrast to lymphoid-resident DCs, whose development is hardly influenced by GM-CSF, migratory DC development requires GM-CSF. CLP indicates common lymphoid progenitor; lin$^-$Flt3$^+$, lineage$^-$Flt3$^+$ hematopoietic progenitor; CMP, common myeloid progenitor; MDP, macrophage and DC progenitor; CDP, common DC progenitor; pre-DC, precursor DC; mono, monocytes; pDC, plasmacytoid DC; cDC, conventional DC; intDC, interstitial DC; LC, Langerhans cell; and TIP-DC, TNF-α and inducible nitric-oxide synthase-producing DC.

but also unconventional DC precursors, such as monocytes. This influences the relative contributions of certain DC subsets as well as the immunogenicity of the DCs generated. These functions are very different from the actions of fms-related tyrosine kinase 3 ligand (Flt3L), a cytokine that supports the development of most DC subsets, with a relatively tolerogenic functionality.[2,20] Although in the past Flt3L-induced DC development under steady-state conditions has attracted more attention than GM-CSF–induced development induced by inflammation, in the light of the continuous exposure to pathogens, inflammation-induced DC development appears highly relevant. Taken together, GM-CSF appears a significant factor in the maintenance of DC homeostasis under both steady-state and inflammatory conditions.

GM-CSF–activated signaling modules regulating DC development

The GM-CSFR contains 2 distinct subunits, the GM-CSF–specific α-chain (GM-CSFRα; CD116) and the common β-receptor (βc; CD131), which is shared between the GM-CSFR, the IL-3 receptor, and the IL-5 receptor.[21,22] Downstream signaling cascades are primarily induced through interaction of effector proteins with the βc subunit. Signaling is initiated by the cytoplasmic tyrosine kinase janus kinase 2 (JAK2), which then acts on various downstream proteins (Figures 2 and 3).[21-24] The principle signaling modules activated include the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, the mitogen-activated protein kinase (MAPK) pathway, and the phosphatidylinositol 3-kinase (PI3K) pathway (Figures 2 and 3). In addition, canonical NF-κB transcription factors are activated. Although NF-κB activation could occur secondary to the activation of signaling proteins, such as STAT5, PI3K, or PKB, or depend on GM-CSF–induced secretion of other factors initiating canonical NF-κB activation, activation independent of such mechanisms has been reported. βc-bound TRAF6 causes NF-κB translocation through induction of IκBα degradation[25] and direct interaction between IκB kinase (IKK) and GM-CSFRα resulting in IκBα degradation has been described,[26] demonstrating that canonical NF-κB activation can be a direct consequence of GM-CSFR engagement (Figures 2 and 3). To elucidate the role of these signaling cascades in DC development, these pathways have been manipulated in DC progenitors or precursors and their DC generating ability has been evaluated.

JAK/STAT

Conditional *JAK2* deletion in adult mice significantly reduced their splenic DC numbers.[27] In addition, murine *JAK2*$^{-/-}$ bone marrow progenitors generated decreased numbers of conventional DCs after culture in the presence of GM-CSF.[27] Despite their status as direct JAK substrates, studies investigating the function of STAT proteins for DC development are relatively scarce. In DCs and their progenitors, GM-CSF–induced activation of STAT1, STAT3, STAT5, and STAT6 has been proposed, but only the roles of STAT3 and STAT5 have been further evaluated.[28-32] STAT3 has proven to be both required and instructive in Flt3L-induced murine DC development but appeared dispensable in GM-CSF–driven DC differentiation cultures.[30,33] With this in mind, the normal DC numbers found in the blood of hyper IgE-syndrome patients carrying *STAT3* mutations[34] could either result from residual STAT3 activity or from compensation by other STATs. For example, in GM-CSF–induced murine bone marrow-derived DC differentiation cultures, STAT5 has been shown to have the capacity to compensate for loss of STAT3 activity.[28-30] Although STAT3 may also be able to compensate for STAT5 deficiency,[29] STAT5 and not STAT3 has a key function in the induction of DC development driven by GM-CSF.

STAT5 has been suggested to support the differentiation of human migratory DCs and murine bone marrow–derived conventional DCs.[28,29,32,35,36] In a recent study, we showed that GM-CSF–induced interstitial DC differentiation from human CD34$^+$ hematopoietic progenitor cells (HPCs) requires STAT5 activity.[32] However, inhibition of STAT5 promotes the development of CD34-derived

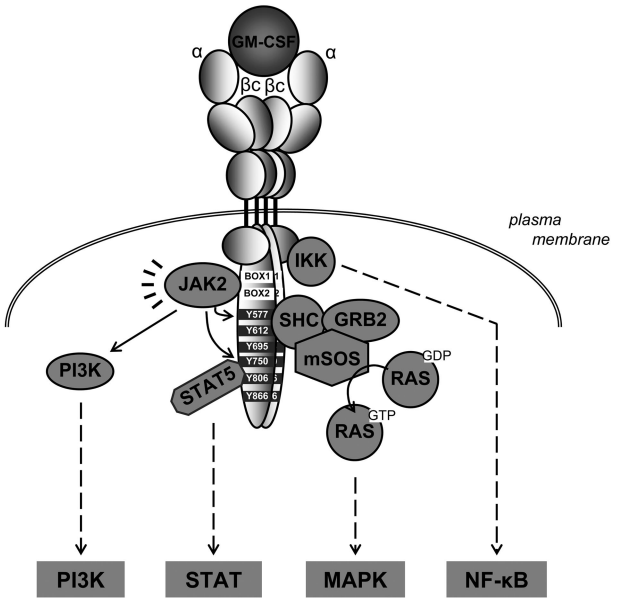

Figure 2. The GM-CSF receptor: initiation of signal transduction. In the absence of GM-CSF, preformed βc dimers as well as single GM-CSFRα chains are present on the plasma membrane. Low affinity binding of GM-CSF to GM-CSFRα causes association with a βc dimer. This leads to the formation of a hexamer of 2 GM-CSF molecules, 2 GM-CSFRα chains, and 2 βc chains, and induces high-affinity GM-CSF/GM-CSFRα binding. Two of these hexamers then dimerize. This brings the βc subunits in close enough proximity to enable JAK2 transactivation, which initiates downstream signaling. Activated JAK2 phosphorylates several tyrosine domains on βc (Y577, Y612, Y695, Y750, Y806, and Y866), which subsequently serve as docking sites for a variety of proteins. Phosphorylation of recruited STATs results in their activation and serves as start of signaling. Activation of the MAPK pathway is initiated by recruitment of SHC to Y577. Its subsequent phosphorylation allows interaction with GRB2 and mSOS, after which the activation of RAS is catalyzed. Recruitment and activation of PI3K has also been suggested to depend on βc residue phosphorylation, and its activation is promoted by JAK2-mediated phosphorylation of PI3K. Finally, GM-CSF activates canonical NF-κB transcription factors. Activation of the IKK complex as a direct consequence of GM-CSFR engagement has been reported, but the proteins involved and the complete chain of events remain to be elucidated.[21-26]

Langerhans cells,[32] demonstrating subset-specific regulation within the migratory lineage. Furthermore, ectopic STAT5 activation reduced the in vitro development of murine conventional DCs[37] and abrogated commitment of human CD34$^+$ HPCs to both the interstitial DCs and the Langerhans cell linage.[32] In contrast, elevated STAT5 activity levels promoted terminal differentiation of already committed human CD34-derived pre-interstitial DCs and pre-Langerhans cells.[32] These data show that the levels and timing of STAT5 activation need to be carefully regulated to ensure DC development. Whereas the activity levels induced by GM-CSF allow the development of migratory and conventional DCs, GM-CSF–induced STAT5 activity inhibits commitment of murine bone marrow progenitors to the plasmacytoid DC lineage.[14,28,29] In contrast, GM-CSF treatment of interferon-producing cells promotes, rather than restrains, terminal differentiation,[15] again indicating the importance of timing. Thus, STAT5 does not act in a simple on-off binary manner and the required STAT5 activity levels differ between DC subsets and differentiation stages.

PI3K/PKB

Spleens of mice lacking the p85α subunit of class IA PI3K contain DCs, but the numbers have not been compared with control mice.[38] PI3K generates PtdIns(3,4,5)P_3, which acts as a second messenger to induce PI3K-dependent signaling (Figure 3). The PtdIns(3,4,5)P_3 phosphatases PTEN and SHIP prevent PI3K-induced activation of downstream signaling. Increased numbers of CD8α$^+$ DCs were observed in spleens of irradiated mice reconstituted with *PTEN*$^{-/-}$ bone marrow progenitors, and spleens of mice with a DC-specific *PTEN* deletion also showed an amplified CD8α$^+$ DC population.[39] Together with the increased CD11c$^+$ splenic DC numbers described for *SHIP*$^{-/-}$ mice,[40] these data indicate that PI3K activity supports DC development. A major target of PI3K-dependent signaling is protein kinase B (PKB/c-AKT), a protein whose

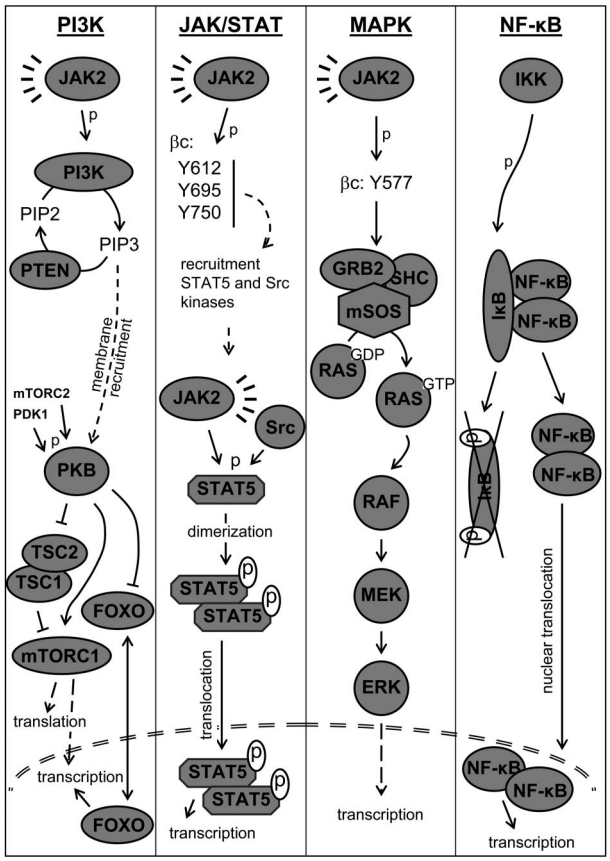

Figure 3. GM-CSF–activated signaling modules. PI3K[93-95]: GM-CSF induces activation of the class IA PI3Ks, which consist of 2 subunits: a catalytic subunit, p110, and a regulatory subunit, p85. Activity of PI3K is promoted by JAK2-mediated phosphorylation of p85. On activation, PI3K functions mainly through the generation of PtdIns(3,4,5)P_3 (PIP3), an activity counteracted by phosphatases PTEN and SHIP. PIP3 acts as a second messenger, regulating a large variety of downstream targets, including protein kinase B (PKB; also called c-AKT). By recruiting PKB to the plasma membrane, PIP3 enables its activation through phosphorylation by PDK1 and mTORC2. Activated PKB regulates many targets, including the FOXO transcription factors, the TSC1/TSC2 complex, and the mTOR complex 1 (mTORC1). Like FOXO, mTORC1 acts through the regulation of transcription, but its main function is in the regulation of protein translation. JAK/STAT[90]: STAT proteins as well as Src kinases are recruited to βc by their SH2 domains that interact with phosphorylated Y612, Y695, and Y750. The STATs are primarily phosphorylated by JAK2, but kinase activity of the Src kinases has also been reported. STAT phosphorylation at a conserved tyrosine residue alters their conformation, which allows the formation of homodimers or heterodimers with DNA-binding and transcription-regulating ability. These dimers then translocate to the nucleus where they act as functional transcription factors. MAPK[59]: Although the human MAPK family includes at least 11 members subdivided into 6 groups, the principle MAPK pathway activated by the GM-CSF receptor is the MEK/ERK pathway. Recruitment of mSOS to the SHC/GRB2 complex enables mSOS to catalyze RAS activation. Formation of active GTP-bound RAS from inactive GDP-bound RAS leads to the successive activation of RAF, MEK, and ERK. On activation, ERK expresses kinase activity toward a variety of cytoplasmic molecules and nuclear proteins, which in turn regulate gene expression. NF-κB[127]: In resting cells, canonical NF-κB dimers consisting of NF-κB/Rel family members RelA, c-Rel, p50, and/or p52 are retained in the cytoplasm by binding to inhibitor of NF-κB proteins (IκBs). Activation is achieved through the IKK complex, which phosphorylates IκB proteins. These are subsequently ubiquitinated and finally degraded, enabling nuclear translocation of canonical NF-κB dimers.

activation involves phosphorylation by PDK-1. DC populations of mice expressing approximately 10% of normal PDK-1 were unaffected,[41] suggesting that either PKB is not required for DC development or the residual PDK1 activity maintained PKB activity at sufficient levels. Bone marrow of β_2-microglobulin$^{-/-}$NOD/SCID mice transplanted with human CD34$^+$ HPCs expressing a constitutively active PKB mutant contain increased human BDCA-1$^+$ DCs compared with mice transplanted with control HPCs,[42] indicating a supportive role for PKB in DC development. Although the PI3K/PKB axis has many downstream effectors, impaired generation of DC after in vivo administration of rapamycin, a pharmacologic inhibitor of mammalian target of rapamycin (mTOR), to mice,[43] indicates that activity of PKB target mTOR complex 1 (mTORC1) is required. Although it has been suggested that the unaffected CD11c$^+$ DC numbers in the blood of kidney transplant patients treated with rapamycin triple therapy indicate mTOR-independent regulation of human blood DC, this study lacks the proper control group.[44]

In vivo experiments clearly demonstrate the importance of PI3K, PKB, and mTORC1 activity in DC homeostasis, but in vitro experiments were required to specifically establish the role of PI3K-PKB-mTOR signaling during GM-CSF–induced DC development. Pharmacologic inhibition of PI3K or mTOR inhibits in vitro GM-CSF–driven human DC development from monocytes[44-48] and hematopoietic progenitors[42,46] because of reduced proliferation and survival of DC precursors. Increased signaling improves survival of DCs and their precursors, and these cells can even be rescued from GM-CSF deprivation-induced apoptosis by ectopic activation of this pathway.[42,49,50] Although the survival of most DC lineages appears to be supported by PI3K-dependent signaling, important differences between distinct subsets and differentiation phases have been observed. For instance, human CD34-derived pre-interstitial DCs and pre-Langerhans cells require PI3K activity to survive, but the survival of terminally differentiated DCs of these lineages is independent of PI3K or mTOR activity.[42,46] Conversely, whereas monocyte survival was found to be resistant to inhibition of PI3K or mTOR, the survival of human monocyte-derived DCs requires activity of this pathway.[46,48] Contradictory experiments were shown in a recent study that reported apoptosis of monocytes 2 days after rapamycin administration.[44] These discrepancies may further emphasize the very specific requirements of distinct subsets at specific differentiation stages.

Although DC numbers are strongly reduced in cultures where PI3K or mTOR is inhibited, the cells show a normal DC phenotype in most studies. However, despite their DC phenotype, these cells are functionally impaired,[42,44,51,52] indicating that the acquisition of full DC function requires activation of this pathway. Besides mTORC1, other PKB-regulated effectors may be involved in DC differentiation. Activity of glycogen synthase kinase-3β (GSK-3β), which is negatively regulated by PKB-dependent phosphorylation, appears required to avoid human monocyte-to-macrophage differentiation in monocyte-derived DC differentiation cultures.[53] However, GM-CSF–induced murine bone marrow-derived conventional DC differentiation was found to be independent of GSK-3β.[54] Although PKB activity levels may affect phenotypic differentiation of some DC subsets, the greatest significance of the PI3K/PKB pathway lies in functional differentiation and promoting DC numbers by ensuring sufficient expansion and survival.

MAPK

Few studies have critically evaluated the role of MAPK signaling during DC development. Although there are several MAPK family members regulating distinct signaling events, the MEK/ERK pathway is the only one directly activated by the GM-CSFR (Figures 2 and 3). In a recent study, conventional DCs derived in vitro from murine *ERK1*$^{-/-}$ bone marrow progenitors were found to show increased surface expression of activation markers and enhanced T-cell stimulation,[55] suggesting that ERK1 negatively influences functional differentiation. Effects on DC yields or typical DC markers were not detailed. Although these data could indicate that ERK is not required for DC development, compensation by the ERK2 isoform provides an alternative explanation. Pharmacologic inhibition of MEK or ERK abrogates both differentiation and survival during human monocyte-derived DC development.[47,48] Although further direct evidence is limited, an association between loss of MEK/ERK activity and reduced DC development has been reported by several groups.[56-58] Interestingly, activation of p38 MAPK[59] by stimuli, such as tumor-secreted factors or Toll-like receptor triggering, can impair GM-CSF–driven DC differentiation, whereas differentiation is improved by pharmacologic inhibition of p38 MAPK.[47,60,61] Thus, GM-CSF can activate the MEK/ERK signaling module to promote DC development, whereas other factors may use p38 MAPK to modulate this. However, the significance of MAPK signaling remains poorly defined, and additional research is required. Considering the key function MAPK proteins have in regulating survival, proliferation, and differentiation of a large variety of cell types, an important role of this pathway in DC development is to be expected.

NF-κB

Individual knockout of the canonical NF-κB proteins RelA, c-Rel, or p50 had no effect on DC populations present in mouse spleen, but spleens of mice with a combined RelA and p50 deficiency contained reduced CD11c$^+$ DCs.[62] Moreover, *RelA*$^{-/-}$*p50*$^{-/-}$ hematopoietic progenitors were unable to generate DCs after adoptive transfer, and their DC differentiation ability in GM-CSF–driven cultures was strongly impaired.[62] A requirement for canonical NF-κB transcription factor activity has also been demonstrated for human migratory DC differentiation from monocytes or CD34$^+$ hematopoietic progenitors in GM-CSF–supplemented cultures.[63-65] Besides supporting the conclusion that canonical NF-κB is required for mouse and human GM-CSF–driven DC development, these in vitro studies allowed separate evaluation of the different aspects of DC development.

Pharmacologic- or viral transduction-mediated NF-κB inhibition strongly reduces cell yields in GM-CSF–driven DC differentiation cultures from human monocytes[64] and human or mouse hematopoietic progenitors.[62,64] The survival of both DC precursors[63,64] and differentiated DCs[64,65] was shown to depend on intact canonical NF-κB activity. Cells derived from GM-CSF–supplemented murine *RelA*$^{-/-}$*p50*$^{-/-}$ bone marrow progenitor cultures had a typical conventional DC morphology,[62] suggesting that differentiation was unaffected. However, canonical NF-κB inhibition in human monocytes and hematopoietic progenitors impaired their acquisition of a DC phenotype,[64,66] indicating that at least for some subsets canonical NF-κB activity is required for differentiation. In particular, human monocyte-derived and CD34-derived interstitial DC differentiation was inhibited in the presence of pharmacologic NF-κB inhibitors,[64] whereas CD34-derived pre-Langerhans cells developed despite NF-κB inhibition.[64] However, terminal differentiation of pre-Langerhans cells was hindered by introduction of an IκBα super-repressor.[66]

GM-CSF–induced canonical NF-κB activation thus appears crucial to ensure differentiation and survival of DC precursors.

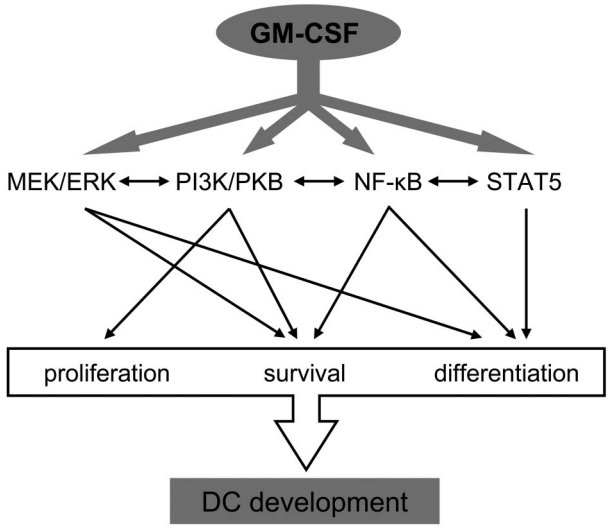

Figure 4. GM-CSF regulates DC development through an integrated molecular network. The signaling modules activated by GM-CSF from an integrated network with overlapping and separate functions that enable an adequate response to a wide range of situations. The currently known functions of the specific cascades in the regulation of GM-CSF–induced DC development are shown. Of the signaling proteins contributing to GM-CSF–driven DC development, JAK2-activated STAT5 is the clearest regulator of differentiation. The main role of the PI3K-PKB signaling module is in promoting expansion and survival of DC precursors rather than their differentiation, although its activity is also required to generate DCs with full functionality. Finally, activation of MEK/ERK and canonical NF-κB transcription factors is associated with differentiation and survival.

Interestingly, despite their incomplete DC phenotype, antigen uptake, activation-induced costimulatory molecule expression, and allogeneic T-cell stimulation by human monocyte-derived DCs generated in the presence of NF-κB inhibitors was comparable with control monocyte-derived DCs.[64] However, their cytokine production abilities might be affected.[64] Similarly, conventional DCs generated in vitro from *p50*$^{-/-}$*c-Rel*$^{-/-}$ murine bone marrow progenitors had a reduced ability to produce IL-12.[62] Continued NF-κB inhibition after differentiation could be responsible for impaired functionality because DC activation induced by pathogenic stimulation, T-cell signals, or inflammatory cytokines other than GM-CSF is strongly dependent on canonical NF-κB activity.[67] Although more detailed investigation is required, it is probable that the activation of canonical NF-κB transcription factors during DC differentiation affects the functionality of the cells generated.

GM-CSF regulates DC development through an integrated molecular network

Manipulation of the activity of central signaling proteins and the evaluation of the consequences for DC development has led to the identification of specific functions for the various GM-CSF–activated signaling pathways described above (Figure 4). However, although in these studies the distinct signaling cascades are often regarded as separate entities, this is mostly not the case because the different pathways share signaling proteins, upstream initiators of signaling, and proteins regulating negative feedback (Table 1). Therefore, manipulation of one signaling module will probably also affect others, meaning that observed effects on DC development may be accounted for by other pathways than the canonical pathway to which the targeted protein belongs. This realization stresses the need to carefully evaluate the available data. The difficulty this causes in the interpretation of some studies is highlighted by the finding that, although PI3K activates NF-κB (Table 1), canonical NF-κB but not PI3K regulates phenotypic human monocyte-derived DCs and CD34-derived interstitial DC differentiation[42,46,64] (Figure 4). This discrepancy is probably explained by PI3K-independent NF-κB activation. However, it seems prudent to keep interpathway regulation in mind when trying to assemble a reliable picture of the molecular aspects of GM-CSF–driven DC development.

Besides processes specifically regulated by distinct signaling cascades, other processes, such as DC survival, are regulated by several GM-CSF–activated signaling modules (Figure 4). This broad regulation is helpful in ensuring DC survival under diverse circumstances, for example, when GM-CSF–induced survival signaling through a specific pathway is hindered by additional environmental stimuli. Furthermore, in a recent model of GM-CSF–induced hematopoiesis, low GM-CSF concentrations were suggested to only activate PI3K, and concentrations of more than or equal to 10pM were required to activate MAPK and JAK/STAT signaling and to induce phosphorylation of PKB.[24] Because survival could be mediated through PI3K activated by low GM-CSF concentrations, hematopoietic cell survival is ensured under all circumstances, whereas additional PKB activation and MAPK and JAK/STAT signal transduction seem required to induce proliferation.[24] Although the relevance of this finding for GM-CSF–induced DC development requires further evaluation, it is interesting to note that the relative contribution of the various modules may not be equal under all circumstances. Thus, DC development in response to GM-CSF is regulated through an integrated network of signaling pathways that act separately but also influence each other's activity. The combination of overlapping and separate

Table 1. Interdependency of distinct signaling pathways

Protein	Substrate	Action
JAK2	STATs	Signaling initiation
	MAPK	Signaling initiation
	PI3K	Signaling initiation
	IκB	Degradation (phosphorylation)
PDK1	PKB	Activation (phosphorylation)
	IKKβ	Activation (phosphorylation)
RAS	RAF	Activation (phosphorylation)
	PI3K	Activation
PP2A	IKK	Inhibition (negative feedback)
	PKB	Inhibition (negative feedback)
SOCS1	STAT5	Inhibition (negative feedback)
	NF-κB	Inhibition (negative feedback)
PIAS1	JAK2	Inhibition (negative feedback)
	RelA	Inhibition DNA binding
PI3K	PKB	Activation (indirect)
	NF-κB	Activation (phosphorylation)
PKB	mTORC1	Activation (phosphorylation and indirect)
	TSC2	Inhibition (phosphorylation)
	RelA	Enhanced activity (phosphorylation)
	IKKβ	Activation (indirect)
p38 MAPK	NF-κB	Regulation DNA binding
ERK	TSC2	Inhibition (phosphorylation)
NF-κB	SOCS1	Increased production
STAT5	NF-κB	Activation (phosphorylation)
IKKβ	IκBα	Degradation (phosphorylation)
	TSC1	Inhibition (phosphorylation)

Although the various signaling cascades regulated by the GM-CSF receptor are often regarded as separate entities, they are actually interrelated, sharing proteins regulating their activation or inhibition, or even directly influencing each other. Proteins involved in more than 1 signaling cascade are listed, together with the proteins they regulate and the actions they express toward them. If 1 of the regulated proteins is regarded as a principle target, this protein is underlined.

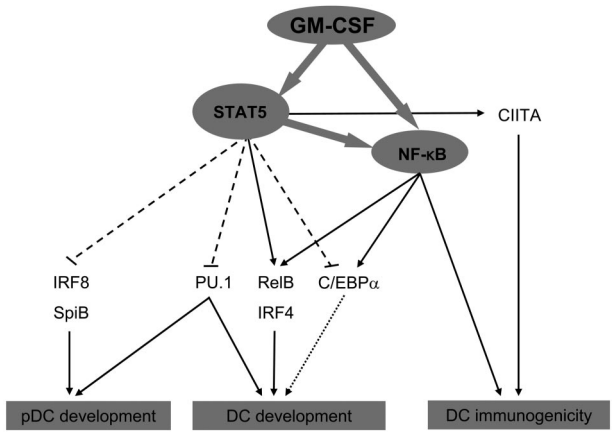

Figure 5. GM-CSF–induced regulation of the DC transcriptional program mediated through STAT5 and canonical NF-κB. STAT5 and canonical NF-κB transcription factors can directly regulate the DC transcriptional program. Regulation of RelB, IRF4, and PU.1 expression not only promotes DC differentiation in general but may also influence subset distribution among the DCs generated. The role of C/EBPα is also subset-specific, promoting development of interstitial DCs through potentiating the commitment of multipotent hematopoietic progenitors to the granulocyte/macrophage lineage, but inhibiting Langerhans cell development. The inhibitory actions of STAT5 toward IRF8 and SpiB explain the negative impact GM-CSF has on the plasmacytoid DC lineage, whereas effects on genes involved in DC functionality may promote the immunogenicity of DC differentiated with GM-CSF.

functions of the distinct signaling proteins enables an appropriate response to a wide range of situations.

Finally, although GM-CSF is often associated with DC development, GM-CSF–activated signaling events could support the differentiation of other hematopoietic lineages. The GM-CSFR is expressed at high levels by (developing) macrophages and granulocytes in addition to DCs[21]; and, for example, GM-CSF–induced PI3K/PKB-dependent survival or progenitor expansion could support the development of these lineages. Furthermore, transcription factors, such as STAT5, promote differentiation of other myeloid cells besides DCs, such as granulocytes and erythrocytes.[68] However, the principle cytokines inducing STAT5 activation in these lineages include G-CSF, IL-5, and erythropoietin rather than GM-CSF, and to induce differentiation of these lineages additional actions of these cytokines are required. Together, the actions of the complete GM-CSF–induced signal transduction network support the generation of DC.

Molecular regulation of GM-CSF–driven DC development

Regulation of DC differentiation and subset distribution

Cell fate decisions in the hematopoietic system are initiated by cytokine-induced signal transduction and established by the resultant actions of transcription factors that regulate lineage commitment and differentiation of hematopoietic progenitors. DC differentiation is driven by a specific set of regulatory transcription factors, as reviewed elsewhere,[2,69] but how the intracellular signaling pathways regulating DC development control this transcriptional program remains ill-defined. GM-CSF–activated canonical NF-κB and STAT5 transcription factors directly regulate genes involved in DC differentiation (Figure 5). For example, NF-κB p50 induces transcription of *C/EBPα*.[70] Although this transcription factor may promote the development of interstitial DCs through potentiating the commitment of multipotent hematopoietic progenitors to the granulocyte/macrophage lineage, C/EBPα activity inhibits the development of Langerhans cells.[71,72] The promoter of *IRF4*, a gene involved in subset-specific DC development, also contains several putative NF-κB binding sites.[73] RelA, c-Rel, and p50 bind *IRF4* promoter elements during human monocyte-derived DC differentiation.[73] For murine DCs, IRF4 expression was shown to be required for GM-CSF–driven conventional DC differentiation in vitro, whereas spleens of *IRF4*$^{-/-}$ mice lack CD8α$^-$ DCs, but CD8α$^+$ DCs are unaffected and numbers of plasmacytoid DCs are only modestly reduced.[74,75] Another subset-specific regulator is RelB. Activation of this noncanonical member of the NF-κB family is induced independently from the canonical NF-κB signaling pathway, but interaction between canonical and noncanonical NF-κB proteins has been described.[76-78] Similar to IRF4, RelB is crucial in the differentiation of CD8α$^-$ splenic DCs,[79] and within the human immune system RelB has been shown to be required for interstitial DCs but not Langerhans cell differentiation.[66] Besides affecting lineage decisions, canonical NF-κB–mediated regulation of RelB may contribute to the development of DCs with a more activated phenotype because this protein has also been associated with DC immunogenicity.[80] Finally, NF-κB is known as a major inducer of inflammatory cytokine and costimulatory molecule expression in terminally differentiated DCs[67] and its activation by GM-CSF may therefore affect the functions of GM-CSF–differentiated DCs.

In addition to NF-κB, STAT5 has been suggested to influence subset-specific DC development through induction of IRF4 and RelB mRNA expression, although this may occur indirectly.[29,36] More direct may be its negative effect on PU.1 and C/EBPα.[32,81,82] For these proteins, expression levels have been suggested to affect hematopoietic lineage choices,[71,83,84] and STAT5-mediated down-regulation of PU.1 and C/EBPα[32,81,82] may be involved in maintaining the correct expression levels submissive for DC development. Furthermore, regulation of expression levels could affect DC subset decisions. In an in vitro system simultaneously generating human Langerhans cells and interstitial DC from CD34$^+$ hematopoietic progenitors, Langerhans cell development is favored by inhibiting C/EBPα activity or enhancing PU.1 expression levels, at the cost of interstitial DC development.[71,85] In addition, murine CD8α$^-$ but not CD8α$^+$ splenic DCs are dependent on PU.1 for their development.[86] Other mechanisms by which STAT5 may regulate subset distribution include the inhibition of IRF8 and SpiB mRNA expression.[29] These transcription factors are mainly associated with plasmayctoid DC development,[2,69] and the inhibitory actions of STAT5 toward these genes could explain the negative impact of GM-CSF on the plasmacytoid DC lineage. Indeed, spleens of IRF8 null mice show specific reductions in plasmacytoid DCs and CD8α$^+$ DCs.[75] However, human subjects with IRF8 mutations resulting in strongly reduced IRF8 activity show general loss of DCs in peripheral blood,[87] implicating a broader effect of IRF8 on human DC development. In addition to regulating subset distribution, STAT5 promotes expression of MHC class II transactivator protein (CIITA),[35] which is essential for transcriptional activity of the *MHC class II* promoter. Because STAT5 also stimulates DNA binding and transcriptional activity of NF-κB,[88] GM-CSF–induced STAT5 activation may increase the intrinsic immunogenicity of DCs generated in the presence of GM-CSF.

The PI3K/PKB and the MEK/ERK module have both been demonstrated to influence NF-κB and STAT5 transcriptional activity,[89,90] but NF-κB/STAT5–independent effects on DC regulatory transcription factors also exist. PKB could affect DC differentiation through inhibition of GSK-3β, which directly inhibits

C/EBPα activity.[91] In addition, direct inhibitory effects of ERK on MHC class II transactivator protein expression have been proposed.[92] In conclusion, GM-CSF–activated JAK2/STAT5 and canonical NF-κB are more directly involved in regulating the DC transcriptional program than PI3K/PKB and MEK/ERK (Figure 5). Through regulation of RelB, IRF4, PU.1, C/EBPα, IRF8, and SpiB, NF-κB and STAT5 not only promote DC differentiation in general but may also influence DC subset distribution. Moreover, by affecting genes involved in DC functionality, STAT5 and NF-κB may increase the immunogenicity of GM-CSF–differentiated DCs.

Downstream effectors regulating proliferation and survival

The only GM-CSF–activated signaling pathway that has been explicitly connected to DC precursor expansion is the PI3K/PKB module (Figure 4).[42] This module generally directs proliferation through transcriptional and translational control,[93-95] but the specific mechanisms used during GM-CSF–induced DC development remain undefined. Although direct evidence is currently lacking, it also appears highly likely that MEK/ERK and JAK2/STAT5 are involved in DC precursor expansion, considering their important role in other hematopoietic lineages.[59,68,90] Knowledge on the molecular regulation of DC survival is little more available. The IKK complex inhibits proapoptotic Forkhead box O (FOXO) transcription factors[96] and activates canonical NF-κB transcription factors that induce the expression of Bfl-1/A1, Bcl2, Bcl-xL, and IAP, proteins involved in antiapoptosis.[97-100] Similarly, MEK/ERK signaling has been shown to regulate the expression of BAD, Bcl-2, and Bcl-XL[22,59]; and although no role for JAK2/STAT5 signaling in the regulation of DC survival has yet been described, considering its widespread control of apoptosis regulators this module likely contributes.[68,90] Although Bcl-2, FOXO1, and particularly Bcl-XL have been associated with DC survival,[101] direct evidence that GM-CSF–activated signaling modules regulate DC survival through modulation of these factors is currently unavailable. In contrast, GM-CSF–induced mTORC1-mediated survival of human monocyte-derived DCs has been shown to depend on regulation of antiapoptotic Mcl-1.[48] In addition, GSK-3β activity, which is inhibited by phosphorylation by PKB, can induce apoptosis by Mcl-1 destabilization.[102] Furthermore, PI3K, PKB, and mTORC1 can regulate FOXO1, BAD, and Bcl-2, which could contribute to the maintenance of DCs.[89] The aforementioned factors involved in DC survival regulation were mainly identified for differentiated DCs. Although tempting, extrapolation of these findings to DC precursors may be inappropriate because of the differential expression of apoptosis regulators at distinct DC differentiation stages.[42] In addition, apoptosis regulators as well as signal transduction pathways regulating survival have been reported to be subset-specific,[42,103] stressing the need to separately evaluate each DC subset. Overall, the molecular control of DC viability and expansion in general, and proliferation and survival during GM-CSF–induced development in particular, is very poorly resolved. Interesting findings on subset- and differentiation stage–specific differences have been reported and will hopefully be defined further in the future.

A molecular explanation for the physiologic differences between GM-CSF– and Flt3L-differentiated DCs

Similar to GM-CSF, Flt3L has a key role in the regulation of DC development, but important differences exist between these 2 cytokines. Whereas deficiency of GM-CSF or the GM-CSFR had only minor consequences on the DCs in mouse lymphoid organs and significantly reduced DC numbers were only observed when analysis was extended to nonlymphoid tissue,[10-13] the importance of Flt3L in DC homeostasis was more obvious. Studies analyzing *Flt3L*$^{-/-}$ or *Flt3*$^{-/-}$ mice described an up to 10-fold reduction in spleen, lymph node, and thymic DCs of different subtypes as well as a reduction in specific early DC progenitors, such as the MDP or CDP, although the latter was not confirmed in all studies.[11,104,105] In contrast, *GM-CSF*$^{-/-}$ mice showed a specific reduction in lymphoid tissue and migratory DCs, whereas plasmacytoid DC numbers were unaffected.[10-13] In addition, inflammation-driven monocyte conversion to murine splenic DCs[3,17] and DC generation during acute inflammatory arthritis and antigen-induced peritonitis[18] were reported to depend on GM-CSF, emphasizing the increasing importance of GM-CSF under inflammatory conditions. In accordance with the data on cytokine and/or receptor deficiency, administration of GM-CSF results in a specific expansion of CD11c^{hi}CD11b^{hi} murine spleen DCs, whereas massive expansion of DCs of all categories has been reported in mice and humans injected with Flt3L.[9,106,107] The specific characteristics of GM-CSF and Flt3L can be explained by their molecular actions. Similar to GM-CSF, Flt3L activates STAT3, PI3K/PKB, and MEK/ERK signaling, but variations in the magnitude of activation of these modules and/or in their relative activity compared with each other may account for the differential DC expansion induced by injection of these cytokines.[2] Major differences include the direct activation of canonical NF-κB and STAT5 by GM-CSF, events that are absent in Flt3L-stimulated cells. Whereas Flt3L supports the development of all DC subsets through activation of STAT3,[30,33] STAT5 and canonical NF-κB transcription factor activity induces a different DC subset-distribution in GM-CSF–dependent cultures (Figure 5). However, although the actions of GM-CSF inhibit plasmacytoid DC development in vitro,[14] plasmacytoid DC numbers were unchanged in *GM-CSF*$^{-/-}$ mice,[11] indicating that the in vivo effects of GM-CSF on plasmacytoid DC development may be limited under steady-state conditions.

Besides their differential effects on the composition of the DC pool, GM-CSF and Flt3L induce functional differences in the DCs they generate. DCs differentiated in vitro in Flt3L-dependent cultures resemble the relatively tolerogenic lymphoid-resident DCs found during the steady state, whereas GM-CSF–differentiated DCs have a more immunogenic phenotype and functionality.[19] Similarly, GM-CSF–driven DC expansion in vivo results in the generation of DCs with a robust immune function prone to the induction of Th1 immune responses against for example pulmonary infections.[108,109] In the treatment of cancer, administration of either Flt3L or GM-CSF resulted in the generation of DCs, but better antitumor responses were observed when GM-CSF was used.[110] The tolerogenic function of Flt3L-DC is further supported by the delayed onset of diabetes in NOD mice and the protection from inflammatory bowel disease in mice in response to Flt3L injection, which was associated with increased DC and regulatory T-cell numbers.[20] Again, a molecular explanation is available for the differences between GM-CSF and Flt3L. In the previous section, GM-CSF–induced activation of STAT5 and canonical NF-κB transcription factors increases the intrinsic immunogenicity of the DCs generated (Figures 5 and 6). In contrast, Flt3L-activated STAT3 inhibits canonical NF-κB activity as well as the expression of MHC class II proteins and costimulatory molecules[111-114] and promotes transcription of *IDO*.[115] These actions account for the tolerogenic function of DCs generated in response to Flt3L[20]

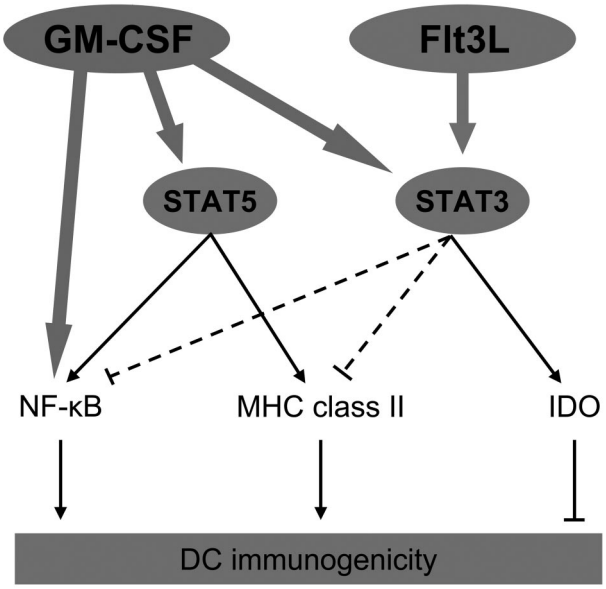

Figure 6. A molecular explanation for the reciprocal effects of GM-CSF and Flt3L on DC immunogenicity. The complementary effects GM-CSF and Flt3L have on the DCs they generate can be explained by their molecular actions. Through activation of STAT3, Flt3L promotes expression of IDO but inhibits canonical NF-κB and MHC class II expression, thereby inhibiting DC immunogenicity. In contrast, GM-CSF activates STAT5 and canonical NF-κB besides STAT3. By promoting NF-κB activity and MHC class II expression, STAT5 overrules the tolerogenic function of STAT3, resulting in the development of more immunogenic DCs.

(Figure 6). Thus, steady-state Flt3L-induced DC development induced through STAT3 yields relatively tolerogenic DCs of all subsets. Under inflammatory conditions, GM-CSF–induced STAT5 and NF-κB activity leads to the generation of specific DC subsets with enhanced intrinsic immunogenicity.

Clinical application

Because of its key role in the generation and function of in particular immunogenic DCs, GM-CSF is currently used in several therapeutic strategies. For instance, inclusion of GM-CSF in mobilization regimens for HSC transplantation has been reported to induce 3 to 10 times higher DC yields during mobilization and to improve DC reconstitution after autologous HSC transplantation.[116,117] Besides aiding immune reconstitution, GM-CSF seems to potentiate antitumor responses by promoting DC generation, migration, and activation when (artificially) produced at the tumor site,[118-120] and promising therapeutic vaccination strategies based on the ex vivo generation and tumor antigen loading of DCs mostly use GM-CSF–based protocols to generate DCs.[121] Finally, GM-CSF is used as potent adjuvant in vaccination strategies for the prevention of hepatitis B virus infection in patient groups with immunization difficulties and for the treatment of hepatitis C virus, human immunodeficiency virus, and fungal or mycobacterial infections.[122]

By addition of GM-CSF, downstream signaling cascades are activated without the possibility to distinguish between those with desired and adverse biologic consequences. Other complicating factors are negative feedback mechanisms and/or the presence of anti-inflammatory signals, for example in the tumor microenvironment, which counteract the effects of GM-CSF. To circumvent these problems, manipulation of the signaling proteins responsible for GM-CSF–driven effects on DCs seems a useful strategy. A potential target is the PI3K-PKB-mTOR signaling module, which could be used to boost DC yields, improve DC survival, and promote DC immunogenicity. Indeed, mouse bone marrow-derived conventional DCs and human monocyte–derived DCs expressing constitutively active PKB or lacking PTEN activity because of siRNA-mediated deletion are highly activated DCs with improved longevity that are able to eradicate tumors more efficiently than control DCs.[49,50] Based on its decisive function in DC lineage commitment,[28,29,32] STAT5 appears a candidate protein for the manipulation of subset specification. Alternatively, the STAT5-regulated factors that are responsible for its effects on lineage decisions, such as PU.1 or IRF8, or the subset-specific regulator RelB[66,79] could be targeted. In mice, silencing of RelB has also been applied to induce tolerance to transplanted hearts and treating autoimmune myasthenia gravis.[123,124] Tolerance has also been promoted through pharmacologic inhibition of mTOR,[52] whereas deletion of tolerogenic STAT3 contributed to the development of immunogenic antitumor immune responses.[125,126] Finally, manipulation of STAT5 and canonical NF-κB transcription factors could be attempted to promote the development of immunogenic DCs.

Although promising, direct targeting of GM-CSF–regulated signaling proteins does not completely resolve the side effects of GM-CSF administration. Specific targeting of the cells of interest remains required, but because a definition of human DC-specific progenitors is lacking, in vivo targeting to early progenitors is currently not possible in humans. Furthermore, interfering environmental signals, negative feedback, and adverse effects caused by interrelation of the different pathways cannot be avoided completely. Despite these remaining challenges, our knowledge on the molecular basis of GM-CSF–induced DC development contributes to the understanding of the specific characteristics of GM-CSF–differentiated DCs, and will hopefully lead to novel strategies to manipulate DC biology for therapeutic purposes.

Concluding remarks

The 4 principle signaling modules activated by GM-CSF, JAK2/STAT5, PI3K/PKB, MEK/ERK, and canonical NF-κB have separate as well as overlapping functions in the regulation of GM-CSF–induced DC development. Although often forgotten, rather than acting as separate entities, these cascades form an integrated network with the activity of one pathway also affecting others. This causes flexibility and enables adequate responses to distinct conditions but complicates the interpretation of experiments in which the activity of a specific signaling protein is manipulated. This is a subject that should be given more attention in the future.

The molecular actions of GM-CSF explain its effects on subset distribution of developing DC and the relatively immunogenic phenotype and functionality of DCs generated by GM-CSF compared with DCs differentiated in response to Flt3L. Further identification of GM-CSF–induced downstream signaling proteins and transcription factors will be of use in the improvement and/or development of DC-based therapies. In addition, further understanding of the ontogenetic background of DCs and of their contribution to immune responses under different pathophysiologic conditions is required. Eventually, this knowledge will enable the use of DCs with tightly controlled functional abilities in novel therapeutic applications.

Authorship

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