Molecular Mechanisms Regulating Human Dendritic Cell Development, Survival and Function

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Molecular Mechanisms Regulating Human Dendritic Cell Development, Survival and Function

Moleculaire mechanismen verantwoordelijk voor
de ontwikkeling, overleving en functie van humane dendritische cellen

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The challenge is in the moment,
the time is always now

James Baldwin

Contents

Abbreviations

Ag	antigen
APAF	apoptotic protease-activating factor
APC	antigen presenting cell
BDCA	blood dendritic cell antigen
CD	cluster of differentiation
CD34-mDC	CD34-derived myeloid DC
CpG	C phosphate G oligodeoxynucleotides
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
ΔΨm	mitochondrial transmembrane potential
eGFP	enhanced green fluorescence protein
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FBS/FCS	fetal bovine serum/fetal calf serum
Flt3L	fms-related tyrosine kinase 3 ligand
4-OHT	4-hydroxytamoxifen
FSC/SSC	forward scatter/side scatter
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBV	hepatitis B virus
HPC	hematopoietic progenitor cell
IFN	interferon
IκB	inhibitor of NF-κB
IL	interleukin
iNGFR	truncated neural growth factor receptor
intDC	interstitial DC
IPC	interferon producing cell
IRES	internal ribosomal entry side
JAK	janus kinase
LC	Langerhans cell
Lox	loxoribine
LPS	lipopolysaccharide
MFI	mean fluorescence intensity
MLR	mixed lymphocyte reaction
mo-DC	monocyte-derived dendritic cell
mono	monocyte
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor-kappa B
NOD/SCID	nonobese diabetic/severe combined immune deficient
pDC	plasmacytoid DC
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B (alternative name: c-AKT)
pre-DC	precursor-DC
PTEN	phosphatase and tensin homolog
RT-MLPA	reverse transcription-multiplex ligation-dependent probe amplification
SCF	stem cell factor
SEM	standard error of the mean
7-AAD	7-aminoactinomycin D
S6K	ribosomal protein S6 kinase
STAT	signal transducer and activator of transcription
TGF	transforming growth factor
TLR	toll like receptor
TNF	tumor necrosis factor

1 GENERAL INTRODUCTION

Dendritic cells in the immune system

A dual function

Dendritic cells (DC) are professional antigen presenting cells (APC) with a dual function in the immune system (Figure 1). On the one hand, these specialized leukocytes are equipped to alert the immune system to invading pathogens or other danger signals. On the other, DC can promote tolerogenic responses, a function believed to be of importance to avoid unlimited immune responses or immunity against self or harmless antigens. To perform these actions, DC continuously sample their antigenic environment, capturing antigens through specific receptor-mediated endocytosis, (macro)pinocytosis or phagocytosis. Recognition of potential danger leads them to instruct and activate antigen-specific adaptive immune cells. Conversely, tolerance is induced by DC that have encountered antigen in the absence of danger signals (1-2).

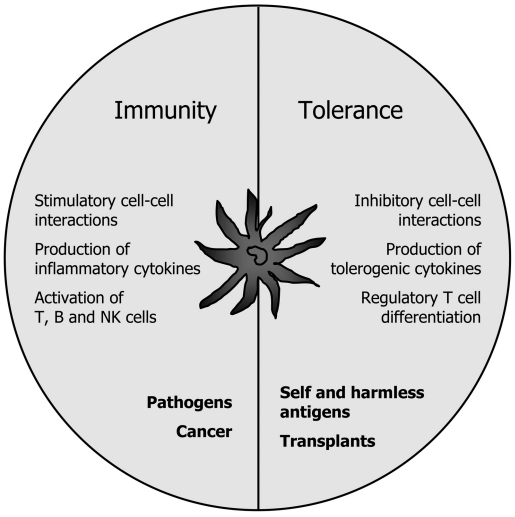


Figure 1. The dual function of DC in immunity
DC can initiate immunogenic as well as tolerogenic immune responses. Induction of immunity is mediated through antigen presentation accompanied by stimulatory cell-cell interactions and production of inflammatory cytokines, resulting in the activation of T, B and NK cells. Antigen presentation to T cells associated with immune inhibitory cell-cell contact and cytokines results in the differentiation of regulatory T cells and tolerance induction. DC can be exploited to generate resistance against pathogens or cancer, but also to induce tolerance to self and harmless antigens or to transplanted organs. By manipulating the function of antigen-presenting DC, DC-based therapies aim to promote or suppress immune responses against selected antigens.

The key role of DC in the induction and regulation of immunity has instigated studies aiming to use them as tools or targets for immunotherapy (Figure 1). Trials aiming to promote anti-tumor immunity using ex vivo antigen-loaded or in vivo targeted DC are currently ongoing and show promising results (3-5). Since reduced DC numbers and functionality were observed in chronic infections, these are another potential target of DC-based vaccines (6-7). In addition to using DC to augment immunity, extensive effort is being put in the development of therapies using tolerogenic DC to prevent rejection of transplanted organs or to restore tolerance in allergies and autoimmune diseases (8-10).

Localization and functionality

DC are found in lymphoid as well as nonlymphoid tissues (2). Peripheral tissue DC remain in the periphery until stimuli, ranging from pathogens or inflammation to disrupted cell-cell interactions, induce their migration through the afferent lymphatics towards secondary lymphoid organs. Since lymphoid tissue also contains so-called lymphoid-resident DC, which remain in the lymphoid tissue throughout their life, tissue-derived and lymphoid-resident DC are both present within these organs. Whereas lymphoid-resident DC represent the majority of lymph node DC under steady state conditions, infection or inflammation can induce a major influx of blood- or tissue-derived DC (11-12). The capability to migrate to lymphoid

tissue distinguishes DC from other professional APC present in peripheral tissue, and contributes to another unique ability, the capacity to activate naïve T cells (see below).
DC operate at the cross-roads between innate and adaptive immunity (Figure 2). At sites of infection, DC can be alerted to danger directly through pattern recognition receptors, or indirectly through signaling from other immune or non-immune cells (13). Activated DC can promote inflammation by secretion of inflammatory cytokines such as IL-6 and TNF-α, whereas production of IL-10 can lessen immunity. The production of CXCR3 ligands supports recruitment of natural killer (NK) cells, and DC-derived cytokines can contribute to the activation of NK and NKT cells (14-17).
In addition to their function in innate immunity, DC can initiate and regulate adaptive immune responses. Antigen captured by DC can be retained unprocessed in cytoplasmic vesicles and subsequently be transferred to naïve B cells to initiate a specific antibody response (18). DC-induced plasma cell differentiation and a role in the induction of antibody class switching have also been described (18-19). Furthermore, the role of DC in T cell immunity is well-established (1-2, 20-21). Next to activating memory T cells, DC have the unique capacity to prime naïve $CD4^+$ and $CD8^+$ T cells. T cell priming requires costimulatory signals in addition to antigen bound to MHC molecules (20). The collection costimulatory molecules expressed and cytokines produced by a DC affect its ability to induce tolerogenic or immunogenic T cell responses (Figure 1). CD86 and CD80 are examples of stimulatory molecules, whereas PD-L1 (B7H1) and PD-L2 reduce immunogenic T cell activation (22). DC-derived cytokines are particularly important in T cell polarization, with for example IL-12, IL-4, IL-23 and IL-10 promoting T helper (Th)1, Th2, Th17 and regulatory T cell differentiation, respectively (2). Similarly, besides $CD8^+$ T cell differentiation towards immunogenic cytotoxic T lymphocytes (CTL), differentiation of $CD8^+$ T cells with a more regulatory function can be induced (23). Finally, besides this role in peripheral tolerance, in the thymus DC support the differentiation of naturally occurring $CD4^+CD25^+$ regulatory T cells and play a role in the negative selection of autoreactive T cells (24-25).

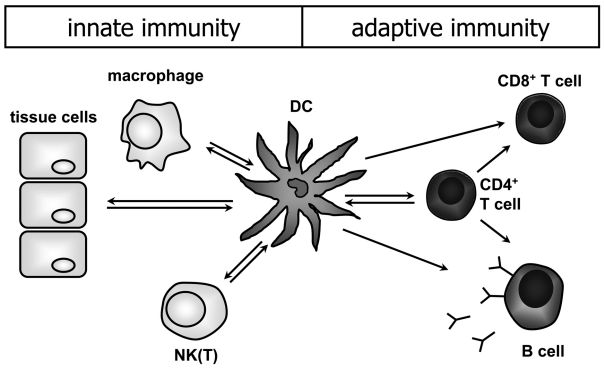


Figure 2. DC at the cross-roads of innate and adaptive immunity
DC are innate immune cells that provide an essential link between the innate and adaptive immune system. Through cytokine secretion and/or cell-cell interaction, DC influence both local tissue cells and innate immune cells such as macrophages, NK cells and NKT cells. In addition, DC have an important function in the induction and regulation of antigen-specific adaptive immune responses. Presentation of captured antigen to T cells is accompanied by stimulatory or inhibitory signals which determine the type of immune response that is initiated. Next to indirectly regulating B cell activity through $CD4^+$ T helper cells, DC also directly interact with B cells. The interaction between DC and other cell types is bi-directional, with many of the above mentioned cells being able to affect DC activity.

Features determining dendritic cell functionality

Dendritic cell subsets

An important determinant of the type of immune response initiated by a DC is the subtype to which it belongs. Based on phenotype, localization, functional differences and ontogeny, a large variety of DC subsets have been identified and new subsets continue to be described.

Comprehensive categorization of the many subsets has been hindered by the low frequency of DC in blood and tissue, and the incompletely resolved relationships between subsets indentified in mice and man. Relatively easy accessibility has resulted in extensive description of DC present in murine organs such as spleen, thymus or gut, whereas research on human DC has focused mainly on subsets accessible in skin and blood, or DC generated in vitro from blood-derived precursors.

In spite of these difficulties, attempts to classify DC have been made. Originally, DC were organized in two main categories, the myeloid and the plasmacytoid lineage (26). Although the term 'myeloid DC' is confusing since plasmacytoid DC may also belong to the myeloid hematopoietic lineage (see below), this name is still used in human DC biology. The lineage$^-$ HLA-DR$^+$ DC identified in human blood are grouped into CD11c$^+$CD123$^-$ myeloid DC and CD11c$^-$CD123^{high} plasmacytoid DC (26-27). Plasmacytoid DC are further characterized by the expression of BDCA-2 and BDCA-4, and express a specific set of innate immune receptors, including the Toll-like receptors (TLR) 7 and 9 (Table 1) (28). Due to their ability to rapidly produce large amounts of type 1 interferon (IFN-1) upon the encounter of viral antigens, plasmacytoid DC are recognized as important anti-viral immune cells. However, their role in immunity is broader than just anti-viral activity, and plasmacytoid DC interact with and influence myeloid as well as lymphoid immune cells through IFN-1-dependent and -independent mechanisms (29-31).

The myeloid DC found in human blood are divided into two subsets by reciprocal expression of BDCA-1 and BDCA-3 (27). Tissue myeloid DC belong to either of two categories, the lymphoid-resident DC or the migratory DC (Table 1) (32-33). Migratory DC act as sentinels for self and non-self antigens in peripheral tissues and migrate towards draining lymphoid organs when stimulated. This class comprises various subsets at different locations, including interstitial DC in skin dermis or mucosal parenchyma, and Langerhans cells in the epidermis of the skin. As opposed to IFN-1-producing plasmayctoid DC, myeloid DC are known for their secretion of IL-12 (28). However, functional differences between the

Table 1. Human DC subsets

Category	Subsets	Phenotype	Localization	Function specificity
Migratory DC	Interstitial DC	CD11c$^+$CD123$^-$ BDCA-1$^+$ CD1a$^-$Langerin$^-$ DC-SIGN$^+$	Peripheral tissues Lymphoid organs	IL-12 production T cell priming B cell activation NK cell activation
	Langerhans cell	CD11c$^+$CD123$^-$ BDCA-1$^+$ CD1a$^+$Langerin$^+$ DC-SIGN$^-$	Epidermis Lymphoid organs	IL-12 production T cell priming
	BDCA-3$^+$ DC	CD11c$^+$CD123$^-$ BDCA-3$^+$DNGR-1$^+$ CD1a$^-$Langerin$^-$ DC-SIGN$^-$	Peripheral tissues Lymphoid organs	IL-12 production Th1 priming Cross-presentation
Lymphoid-resident DC	Conventional (classical) DC		Lymphoid organs	
Plasmacytoid DC	Plasmacytoid DC	CD11c$^-$CD123^{high} BDCA-2$^+$BDCA-4$^+$ CD1a$^-$Langerin$^-$ DC-SIGN$^-$	Blood Peripheral tissues Lymphoid organs	IFN-α production T cell priming NK cell activation

various subsets are apparent. Compared to interstitial DC, Langerhans cells induce primary $CD8^+$ T cell responses more efficiently (34). Interstitial DC are potent inducers of naïve $CD4^+$ T cell differentiation towards follicular helper T cells (34). Together with their ability to directly stimulate B cells (35), this suggests a superior ability to induce humoral immune responses. Finally, the recently described BDCA-3^+ DC is specialized in cross-presenting exogenous antigens to $CD8^+$ T cells (36-37). In addition to intrinsic functional differences, the environment is a major determinant in the functionality of migratory DC. Interstitial DC present in skin, lung, liver or gut display distinct functional features that link to specific needs of the tissue in which they reside (38-41).
The lymphoid-resident DC include the conventional or classical DC described in mouse spleen and other primary and secondary lymphoid organs (33). They show a less activated phenotype than tissue-derived DC, and contrast with migratory DC by collecting and presenting antigen within the same lymphoid organ. Although lymphoid-resident DC are extensively studied in mice, little is known about their human counterparts.

Longevity
DC have a relatively short lifespan. Turnover rates of the distinct subsets have been determined in mice. The half-life of murine lymphoid-resident conventional DC has been estimated at approximately two days, whereas plasmacytoid DC appear longer-lived with a half-life of 8-9 days (42). Under steady state conditions, migratory DC reside in peripheral tissue between one and four weeks, after which they may exist in secondary lymphoid tissue for a limited period (43). Only Langerhans cells have been suggested to persist longer in the epidermis (44).
Besides cell-intrinsic characteristics influencing their longevity, environmental signals have a large impact on DC survival. Cytokines such as Flt3 ligand (Flt3L), GM-CSF and M-CSF promote survival (42, 45), while TGF-β (46) and IL-10 (47) have been shown to induce apoptosis. Recognition of pathogens usually promotes DC viability (48), ensuring their continued existence within lymphoid organs and enabling interaction with adaptive immune cells. Prosurvial factors provided in the lymph nodes, for example by interacting T cells (49), also contribute to this. In addition, mechanisms limiting DC survival, required to prevent unrestricted immune activation, are available. Long term consequences of pathogen recognition include upregulation of pro-apoptotic factors (50-51) and antigen-specific T cells have been suggested to kill antigen-presenting DC in a Fas and perforin-dependent manner (52-54). Together, intracellular and extracellular signals adjust DC longevity to situation-specific requirements, an essential aspect in the regulation of DC functionality. Disturbances of DC lifespan regulation can result in either immune hyperactivation or loss of tolerance.

Activation and maturation
DC can exist in different maturation stages (Figure 3) (1-2, 21). Similar to mature DC, immature DC are fully functional, terminally differentiated DC, and the term ‘immature’ merely refers to the fact that these DC perform a different function than mature DC. Immature DC are well-equipped to capture and process antigens, but less efficient at antigen presentation. Encountering activation signals triggers a short-lived increase in antigen uptake and processing, after which these functions are reduced while MHC class II surface expression is increased (1-2, 21). Since activation also induces lymphoid migration of migratory or plasmacytoid DC, this chain of events ensures that by the time these DC interact with lymphocytes in secondary lymphoid organs, they efficiently present the antigens collected at the time and place migration was induced.
Maturation of DC can be initiated by signals from pattern recognition receptors such as TLRs, NOD-like receptors, RIG-I-like receptors and members of the C-type lectin family (2,

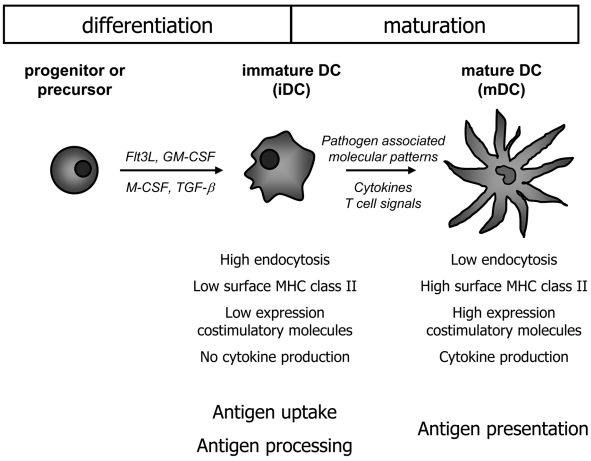


Figure 3. The DC life cycle: differentiation and maturation
The process of DC generation from progenitors or precursors is called differentiation. Fully differentiated cells can exist as immature or mature DC. Due to their high endocytosis capacity but low expression of surface MHC class II, costimulatory molecules and secretion of cytokines, immature DC (iDC) are well equipped to capture and process antigen, while their antigen presenting capacity is low. Activation signals induce maturation, yielding mature DC (mDC) with reduced uptake and processing, but efficient presentation of antigens. This is accompanied by increased expression of costimulatory molecules and secretion of cytokines.

13). Expression of distinct sets of these receptors by specific DC subsets partially determines their capacity to respond to certain pathogens (28). DC maturation induced by pathogenic stimulation is often accompanied by induction of costimulatory molecules, chemokine receptors and cytokines, which together enable the induction of immunity. However, while DC maturation can be induced by various stimuli, not all DC efficiently presenting antigens show similar functionality. DC exposed to inflammatory cytokines such as IL-6, TNF-α and IL-1β upregulate MHC and costimulatory molecules, but lack production of IL-12 (55-56). Similarly, DC triggered by disruption of cell-cell interactions show the typical upregulation of MHC class II, costimulatory molecules and chemokine receptors, but fail to release immunostimulatory cytokines (57). Finally, although IL-12 is elicited by most pathogens, its production is potently boosted upon CD40-CD40L binding during DC-T cell interaction (55, 58). Overall, specific stimuli induce a particular set of costimulatory molecules and cytokines, which will affect the nature of the immune response initiated by the antigen presenting DC.

Dendritic cell development

Ontogeny

The distinct DC subsets, as well as all other cells of the hematopoietic system, are derived from the same early progenitor, the $CD34^+$ hematopoietic progenitor cell (HPC) found mainly in the bone marrow. However, the exact origin and developmental program of DC is only partially understood and the majority of evidence was obtained through mouse experiments. Until recently, plasmacytoid DC were believed to develop from the common lymphoid progenitor (CLP), whereas the common myeloid progenitor (CMP) was thought to give rise to all other DC subtypes (26), hence the term 'myeloid DC' (see above). Contrasting with this view, CLP as well as CMP were shown to have the potential to generate both plasmacytoid and myeloid DC in mice and man (59-61), indicating that the division into myeloid and lymphoid progenitors is not accurate for DC.

Two observations have enabled the identification of a DC-specific progenitor in heterogeneous HPC populations. First, the cell populations within the CMP and CLP that generated DC responded to Flt3L, indicating that Flt3 might be expressed (62). The second clue was the fact that migratory DC, lymphoid-resident DC and the closely related monocytes and macrophages all expressed CX_3CR1 (63-64). This led to the identification of

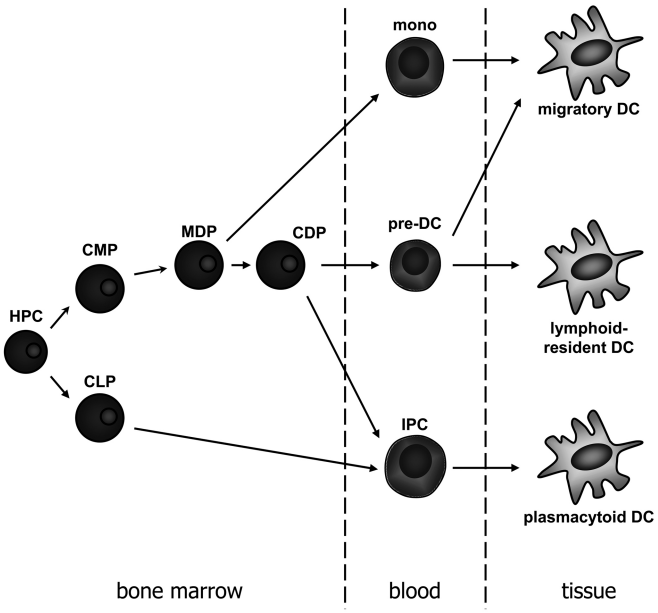


Figure 4. DC ontogeny
All DC subsets are derived from bone marrow-residing hematopoietic progenitor cells (HPC). Downstream of the common myeloid progenitor (CMP), the macrophage and dendritic cell progenitor (MDP) gives rise to monocytes (mono) as well as the common dendritic cell progenitor (CDP). The CDP then generates precursor DC (pre-DC), that egress from the bone marrow and enter the circulation. DC differentiation of monocytes, pre-DC and interferon producing cells (IPC), the pre-DC for plasmacytoid DC, is initiated during migration towards peripheral tissue. In addition, DC development downstream of the common lymphoid progenitor (CLP) has been proposed, in particular for plasmacytoid DC. The presented model is based primarily on data obtained from mouse models.

two specific progenitors originating from the murine CMP (Figure 4). The lineage$^-$Flt3$^+$Sca1$^-$ CD115$^+$CD117^{int}CX$_3$CR1$^+$ macrophage and dendritic cell progenitor (MDP) generates monocytes as well as all dendritic cell lineages (65). It acts as a direct precursor for the common dendritic cell progenitor (CDP), which can only give rise to DC (66-68).
The CDP then generates subset-specific precursor DC (pre-DC) (69). These pre-DC leave the bone marrow and migrate through the blood towards peripheral tissues (Figure 4). Final DC differentiation occurs during blood-to-tissue migration. In addition to pre-DC, monocytes have been described as potential DC precursors, mostly at inflamed sites (70-72). Moreover, DC subsets are not necessarily replenished from bone marrow progenitors throughout life. It has been suggested, for example, that maintenance of Langerhans cell homeostasis is independent of blood-derived precursors, but depends either on self-renewal or on local tissue-resident Langerhans cell precursors (73-74).
Whereas the subsequent stadia of murine DC development from the CMP have become clearer, the role of the CLP remains largely undefined. Although plasmacytoid DC, through MDP and CDP, can develop from the CMP, plasmacytoid DC with DJ rearrangements have also been found (75). In accordance with previous observations, this indicates a lymphoid origin of at least a proportion of plasmacytoid DC. The exact contribution of the CLP to DC maintenance remains to be determined. Furthermore, a human equivalent for the MDP or CDP has yet to be found. In the human hematopoietic progenitor compartment, a multilymphoid progenitor generating T, B and NK cells next to DC was recently described (76). Thus, although the ontogenetic background of DC is beginning to be dissolved, the picture of DC ontogeny has not yet been completed.

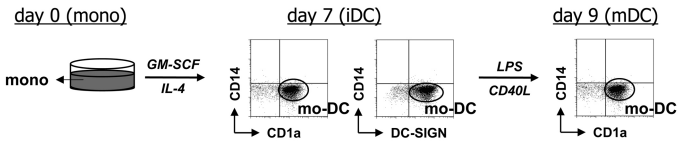
Cytokines maintaining dendritic cell homeostasis
As indicated above, Flt3L appears an important cytokine in the generation of DC. Mice lacking Flt3L show a severe reduction in all DC subsets except Langerhans cells (77-78), and injection or conditional expression of Flt3L results in massive DC expansion in both mice and man (62, 79). The instructive function of this cytokine in inducing in vitro DC development further supports its significance in DC differentiation (80-81). Flt3L is produced

constitutively by stromal cells. Since it is normally consumed by DC, a reduction in the DC population results in elevated serum concentrations, which in turn induces DC replenishment (82). In response to inflammation, the levels of Flt3L are increased through secretion by activated T cells (82). Overall, Flt3L has a central, nonredundant role in the regulation of DC homeostasis and a regulatory loop exists to adjust its levels to ensure sufficient DC development under specific circumstances.
In contrast to the detrimental effects of Flt3L deficiency, loss of GM-CSF or the GM-CSF receptor does not generally affect lymphoid-resident DC under steady state conditions (77, 83). However, without the compensatory actions of Flt3L, GM-CSF appears essential for the maintenance of MDP, CDP and lymphoid-resident and migratory DC (77). Furthermore, even in the presence of Flt3L, a reduction of migratory DC is apparent in GM-CSF$^{-/-}$ mice (77). Its critical function in migratory DC but not lymphoid-resident DC development indicates that the actions of GM-CSF are subset-specific. In accordance, plasmacytoid DC differentiation is not stimulated by GM-CSF, and addition of this cytokine to Flt3L-driven cultures even inhibits the generation of this subset (81). Serum levels of GM-CSF, which is produced by tissue stromal cells and activated T and NK lymphocytes, are usually low but increase in inflammation (45). In addition to its function in the steady state generation of specific DC subsets, GM-CSF is thought to play a key role in DC differentiation under inflammatory conditions (32, 82).
Besides Flt3L and GM-CSF, M-CSF (70) and TGF-β (84-85) have also been associated with DC development, and seem particularly important during Langerhans cell differentiation. Other cytokines, including IL-4, TNF-α and G-CSF, may all skew DC development under different circumstances (32).

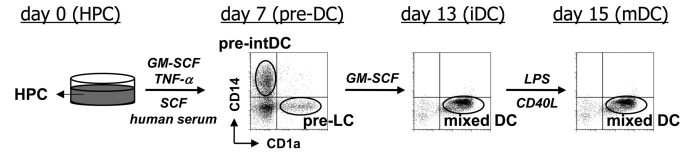
Differentiation, proliferation and survival
DC development from general and subset-specific precursors is a complex process. The acquisition of a DC phenotype and function depends on the subsequent expression or activation of a specific set of transcription factors. However, the development of functional DC involves more than differentiation. Sufficient proliferation of DC progenitors or precursors is required to ensure adequate DC numbers. Finally, survival of DC precursors as well as fully differentiated DC is essential for the generation of functional DC.

Methodologies for the study of human dendritic cell biology
The very low frequencies of DC in blood and tissue combined with the limited accessibility of human material complicate the study of human DC biology. To facilitate human DC research, various in vitro culture systems have been developed (Figure 5). For myeloid DC, differentiation from peripheral blood monocytes is the most widely used model. In addition, interstitial DC and Langerhans cells can be differentiated from less committed CD34^{+} HPC derived from cord blood. CD34^{+} HPC can also generate plasmacytoid DC. In addition to these in vitro systems, human CD34-derived DC development can also be studied in vivo, by xenotransplantation of human HPC into immune-deficient mice (86-88).

Monocyte-derived DC



CD34-derived myeloid DC



CD34-derived plasmacytoid DC

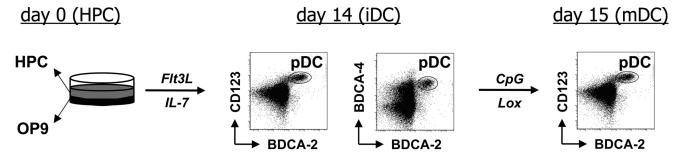


Figure 5. In vitro human DC generation

DC can be generated in vitro from peripheral blood-derived monocytes or cord blood-derived CD34⁺ HPC. Monocyte-derived DC (mo-DC): Monocytes (CD14⁺CD1a⁻DC-SIGN⁻) are cultured in the presence of GM-CSF and IL-4. Immature mo-DC (CD14⁻CD1a⁺DC-SIGN⁺) are derived at day 7. Stimulation with LPS or CD40L induces maturation. CD34-derived myeloid DC: HPC (CD34⁺CD14⁻CD1a⁻) are cultured in the presence of GM-CSF, TNF-α, SCF and human serum. CD14⁺CD1a⁻ interstitial DC precursors (pre-intDC) and CD14⁻CD1a⁺ Langerhans cell precursors (pre-LC) can be distinguished at day 7. Further culture in the presence of GM-CSF induces terminal differentiation. Similar to pre-LC, Langerhans cells (LC) are CD14⁻CD1a⁺. During terminal differentiation, pre-intDC first acquire CD1a and subsequently loose CD14, generating CD14⁻CD1a⁺ interstitial DC (intDC). Thus, a mixed population of CD14⁻CD1a⁺ LC and intDC is present at day 13. Stimulation with LPS or CD40L induces maturation. CD34-derived plasmacytoid DC: CD34⁺ HPC are cultured with OP9 stromal cells, in the presence of Flt3L and IL-7. After two weeks, a population of CD123⁺BDCA-2⁺BDCA-4⁺ plasmacytoid DC (pDC) can be recognized. Stimulation with CpG or Lox induces maturation.

Abbreviations: mono, monocyte; HPC, hematopoietic progenitor cell; pre-DC, precursor DC; iDC, immature DC; mDC, mature DC.

Rationale and scope of the thesis

By translating environmental signals into cellular actions, signal transduction pathways provide the link between extracellular information and intracellular response. However, while the extracellular signals driving DC subset development, survival and function are relatively well understood, the intracellular signaling modules and transcription factors mediating this remain poorly defined. This thesis therefore aims to illuminate the molecular mechanisms regulating human DC biology.

An overview of the intracellular signal transduction pathways activated by GM-CSF and how they regulate differentiation, proliferation and survival of DC progenitors and precursors is provided in **Chapter 2**. While this chapter focuses on GM-CSF-driven DC development, similar pathways are activated by Flt3L, and molecular mechanisms underlying the specific characteristics and functions of GM-CSF-differentiated DC compared to DC populations induced by Flt3L are discussed.

Chapter 3 describes the critical role of the PI3K-PKB-mTOR signaling module in the development and function of myeloid interstitial DC and Langerhans cells. This pathway not only regulates the DC yield by promoting the proliferation and survival of progenitor/precursor cells, but is also involved in the generation of fully functional DC. In contrast to DC progenitors and precursors, the survival of terminally differentiated DC is independent of PI3K-PKB-mTOR signaling.
A possible explanation for the decreased PI3K-PKB-mTOR dependency of interstitial DC and Langerhans cells is given in **Chapter 3-Appendix**. The data presented here provide evidence for a reduced sensitivity to mitochondria-mediated apoptosis in these cells.
In addition to PI3K-PKB-mTOR signaling, **Chapter 4** shows that the development of myeloid CD34-derived as well as monocyte-derived DC requires activation of canonical NF-κB transcription factors. These factors were found essential for the differentiation and survival of progenitors/precursors. In addition, the importance of canonical NF-κB activity in the regulation of DC function is described.
Chapter 5 demonstrates a critical role for STAT5 in controlling CD34-derived interstitial DC and Langerhans cell development. STAT5 is redundant for commitment to the Langerhans cell lineage but required to induce interstitial DC differentiation. Besides subset-specific regulation, the STAT5 activity levels and the timing of STAT5 activation have a major influence on differentiation. Rather than acting in an on-off binary manner, specific requirements exist for the level of STAT5 activation at distinct differentiation stages and in different DC subsets.
Finally, in **Chapter 6**, the PI3K-PKB-mTOR pathway is shown to regulate plasmacytoid DC development and survival. Furthermore, the impaired functionality of plasmacytoid DC from patients chronically infected with hepatitis B virus is associated with reduced activity of this signaling module. The observation that plasmacytoid DC development and function can be boosted by increasing PI3K-PKB activity identifies this axis as a possible target for immunotherapy.
Together, the findings presented in this thesis contribute to the understanding of the regulation of DC subset development, survival and function under different pathophysiological conditions. An integrated view on this matter and its implications for the development of novel treatment strategies to manipulate immunity are discussed in **Chapter 7**.

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2

REGULATION OF DENDRITIC CELL DEVELOPMENT BY GM-CSF: MOLECULAR CONTROL AND IMPLICATIONS FOR IMMUNE HOMEOSTASIS AND THERAPY

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Submitted

Abstract

Dendritic cells (DC) represent a small and heterogeneous fraction of the hematopoietic system, specialized in antigen capture, processing and presentation. They act as sentinels throughout the body, migrate from sites of antigen uptake to sites of cellular interaction, and perform a key role in the induction of immunogenic as well as tolerogenic immune responses. Owing to their limited lifespan, continuous DC replenishment is required. Whereas the importance of granulocyte-macrophage colony-stimulating factor (GM-CSF) in regulating DC homeostasis has long been underestimated, this cytokine is currently considered a critical factor 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 NF-κB. By directing the activity of transcription factors and other cellular effector proteins, these pathways influence differentiation, survival and/or proliferation of uncommitted progenitors and subset-specific precursors, thereby contributing to specific aspects of DC 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 DC compared to DC generated by other cytokines. This knowledge can be used to identify therapeutic targets and develop novel strategies to regulate immunity.

Introduction

Dendritic cells (DC) constitute a crucial and heterogeneous fraction of the hematopoietic system, with an essential role in the induction and regulation of immunity (1). Since DC 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 ontogenetic background of DC subsets belonging to one of the three main categories defined elsewhere (2-4), migratory DC, lymphoid-resident DC and plasmacytoid DC, is depicted in Figure 1. Efficient DC development from hematopoietic stem cells involves proliferation and survival as well as phenotypic and functional differentiation of progenitors with gradually restricted developmental options. Of the various cytokines that have been associated with DC development, fms-related tyrosine kinase 3 ligand (Flt3L) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are the most well-defined (2, 5).

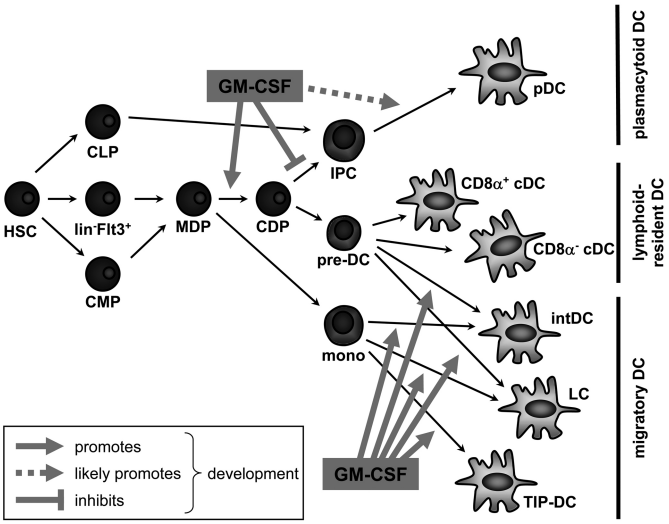

Figure 1. The role of GM-CSF during DC subset development

DC are derived from hematopoietic stem cells (HSC) through gradually restricted precursors. DC subsets can be categorized in three main categories, migratory DC, lymphoid-resident DC and plasmacytoid DC. GM-CSF supports development of the common DC progenitor (CDP). Commitment of the CDP towards the plasmacytoid DC lineage is inhibited by GM-CSF, but terminal differentiation of committed plasmacytoid DC precursors, interferon producing cells (IPC), is likely supported by GM-CSF. In contrast to lymphoid-resident DC, whose development is hardly influenced by GM-CSF, migratory DC development requires GM-CSF.

Abbreviations: HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; lin$^-$Flt3$^+$, lineage$^-$Flt3$^+$ hematopoietic progenitor; CMP, common myeloid progenitor; MDP, macrophage and DC progenitor; CDP, common DC progenitor; IPC, interferon producing cell; pre-DC, precursor DC; mono, monocyte; pDC, plasmacytoid DC; cDC, conventional DC; intDC, interstitial DC; LC, Langerhans cell; TIP-DC, tumor necrosis factor-α and inducible nitric-oxide synthase-producing DC

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

GM-CSF was the first cytokine shown to efficiently promote DC development in vitro (6), and has been used to induce DC differentiation from human monocytes (7), human and mouse hematopoietic progenitor cells (8-11) and the myelomonocytic leukemia cell line MUTZ-3 (12). In accordance, significantly increased DC numbers were found in spleen and thymus of mice injected with GM-CSF or transgenic mice overexpressing GM-CSF (13-15), suggesting that GM-CSF can promote DC expansion in vivo. However, only a marginal reduction in lymphoid organ-resident DC was found in mice lacking GM-CSF or the GM-CSF receptor (GM-CSFR) (15). Because of this apparently minor role during steady state DC

development, instead of GM-CSF, Flt3L was regarded the principle cytokine in the regulation of DC homeostasis. In later studies however, mice deficient for GM-CSF or the GM-CSFR were shown to have reduced numbers of migratory DC in skin and gut (16-18), indicating that development of these subsets requires GM-CSF even under steady state conditions. The threefold decrease in lymph node DC observed earlier (15) likely also reflects a loss in migratory DC. Next to migratory DC, subset-specific effects have been shown for plasmacytoid DC, whose development is negatively affected by GM-CSF (19). Interestingly, while GM-CSF inhibits commitment of general DC progenitors to the plasmacytoid DC lineage (19), it supports the survival and can even initiate terminal differentiation of circulating interferon producing cells (IPC), the direct precursors of plasmacytoid DC (20).
Whereas GM-CSF circulates at very low concentrations in the steady state, its secretion is increased during infection (21-22). Although its role in DC development under steady state conditions may be called subtle, this becomes more pronounced in the presence of inflammation. In vitro GM-CSF-differentiated bone marrow-derived DC resemble tumor necrosis factor-α and inducible nitric-oxide synthase-producing DC (TIP-DC), which are found during infection, rather than lymphoid DC present under steady state conditions (23). Furthermore, inflammation-driven monocyte conversion to murine splenic DC has been suggested to depend on GM-CSF (4, 24). Since GM-CSF specifically regulates distinct DC subsets and can induce DC differentiation from precursors not affected by Flt3L (2), GM-CSF-driven DC development can considerably alter the composition of the DC pool during inflammation. In addition, in contrast to the relatively tolerogenic DC generated by Flt3L, which resemble steady state lymphoid-resident DC, GM-CSF-differentiated DC have a more immunogenic phenotype and functionality (23), a characteristic that likely contributes to their required function during inflammation.
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 prompt differentiation of precursor DC (pre-DC) but also unconventional DC precursors such as monocytes, and may thereby influence the relative contributions of certain DC subsets. Although in the past DC development under steady state conditions has attracted more attention than development induced by inflammation, in the light of the continuous exposure to pathogens, inflammation-induced DC appears highly relevant. Overall, 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 two 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 (25-27). The domains required to induce downstream signaling cascades are primarily provided by the βc subunit. Signaling is initiated by the cytoplasmic tyrosine kinase janus kinase 2 (JAK2), which then regulates downstream proteins (Figures 2 and 3) (25-30). 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). Additionally, independent activation of nuclear factor-kappa B (NF-κB) transcription factors has been described (Figures 2 and 3) (31-33). To elucidate the roles of these signaling cascades in DC development, the DC generating ability of DC progenitors or precursors with manipulated pathway activity has been evaluated.

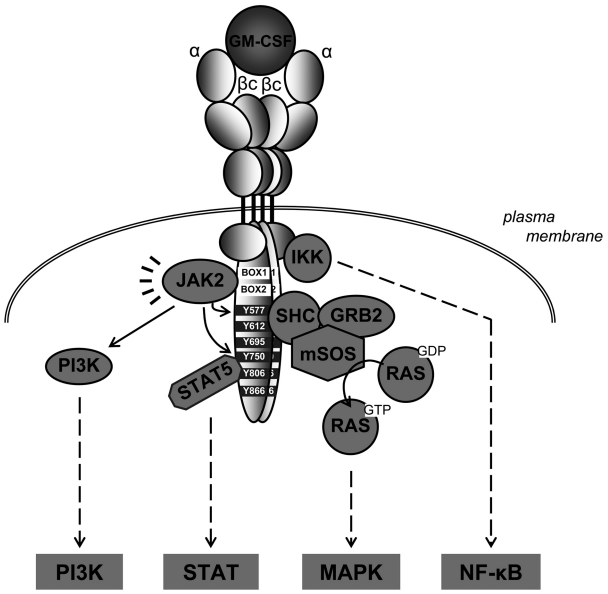

Figure 2. The GM-CSF receptor: initiation of signal transduction

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, which leads to the formation of a hexamer of two GM-CSF molecules, two GM-CSFRα chains and two 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. Binding of the IKK complex to GM-CSFRα as well as βc domains has been reported, but the proteins involved in and the complete chain of events leading to IKK activation remain to be elucidated. (25-33)

JAK/STAT

Consistent with its key role in the initiation of intracellular signal transduction, JAK2 deficiency leads to embryonic lethality. Cell type specific analysis has been facilitated by conditional JAK2 deletion in adult mice, which significantly reduced their splenic DC numbers (34). In addition, murine JAK2$^{-/-}$ bone marrow progenitors generated decreased numbers of DC following culture in the presence of GM-CSF (34). Despite their status as principle JAK substrates, studies investigating the function of STAT proteins for DC development are relatively scarce. In DC and their progenitors, GM-CSF-induced activation of STAT1, STAT3, STAT5 and STAT6 has been suggested (35-40), but only the roles of STAT3 and STAT5 have been further evaluated. 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 (38, 41). In addition, the normal DC numbers found in the blood of hyper IgE-syndrome patients carrying STAT3 mutations (42) could result from residual STAT3 activity, or could indicate 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 (36-38). Although STAT3 may also be able to compensate for STAT5 deficiency (37), 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 DC, MUTZ3-derived DC, and murine bone marrow-derived conventional DC (36-37, 40, 43-46). However, in a recent study we showed that although GM-CSF-induced interstitial DC differentiation from human CD34^{+} HPC requires STAT5 activity, inhibition of STAT5 promotes the development of CD34^{+}-derived Langerhans cells (40), indicating subset-specific regulation within the migratory lineage. Furthermore, ectopic STAT5 activation reduced the in vitro

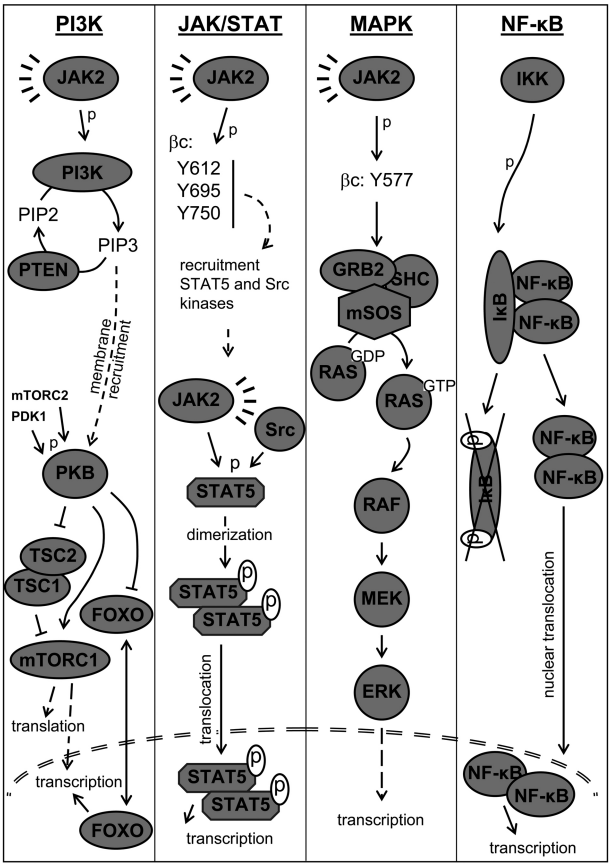

Figure 3. GM-CSF-activated signaling modules

PI3K (113-115). GM-CSF induces activation of the class IA PI3Ks, which consist of two subunits, a catalytic subunit, p110, and a regulatory subunit, p85. Activity of PI3K is promoted by JAK2-mediated phosphorylation of p85. Upon 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 Forkhead box O (FOXO) transcription factors, the TSC1:TSC2 complex and mTOR Complex 1 (mTORC1). Like FOXO, mTORC1 acts through the regulation of transcription, but its main function is the regulation of protein translation.

JAK/STAT (110, 122). 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 phosphylation at a conserved tyrosine residue alters their conformation, which allows the formation of homo- or heterodimes with DNA binding and transcription regulating ability. These dimers then translocate to the nucleus where they act as functional transcription factors.

MAPK (116). Although the human MAPK family includes at least 11 members subdivided into six groups, the principle MAPK pathway activated by the GM-CSFR is the 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. Upon activation, ERK expresses kinase activity towards a variety of cytoplasmic molecules and nuclear proteins, which in turn regulate gene expression.

NF-κB (153). 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 IκB kinase (IKK) complex, which phosphorylates IκB proteins. These are subsequently ubiquitinated and finally degraded, enabling nuclear translocation of canonical NF-κB dimers.

development of murine conventional DC (47) and abrogates migratory DC commitment of human $CD34^+$ HPC (40). In contrast, elevated STAT5 activity levels promote terminal differentiation of already committed human CD34-derived pre-migratory DC (40). These data demonstrate that the levels and timing of STAT5 activation need to be carefully regulated to

ensure development of migratory and conventional DC. Whereas the activity levels induced by GM-CSF allow the development of these DC lineages, GM-CSF-induced STAT5 activity inhibits commitment of murine bone marrow progenitors to the plasmacytoid DC lineage (19, 36-37). In contrast, GM-CSF treatment of IPC promotes rather than restrains terminal differentiation (20), again indicating the importance of timing. Thus, STAT5 does not act in a simple on-off binary manner and different subset-specific requirements for the level of STAT5 activation at distinct differentiation stages are apparent.

PI3K/PKB

Spleens of PI3K deficient mice contain DC, but the numbers have not been compared to control mice (48). PI3K generates PtdIns(3,4,5)P_3 (PIP3), which acts as a second messenger to induce PI3K-dependent signaling (Figure 3). By expressing phosphatase activity towards PIP3, PTEN and SHIP prevent PI3K-induced PIP3-mediated activation of downstream targets. Increased numbers of $CD8^+$ DC 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 $CD8^+$ DC population (49). Together with the increased $CD11c^+$ splenic DC numbers described for $SHIP^{-/-}$ mice (50), these data indicate that PI3K activity supports DC development. A major target of PI3K-dependent signaling is protein kinase B (PKB). PKB activation requires phosphorylation at specific residues by different kinases, including PDK-1. DC populations of mice expressing approximately 10% of normal PDK-1 were unaffected (51), suggesting that either PKB is not required for DC development or the residual PDK1 activity maintained PKB activity at sufficient levels. Bone marrow of β2-microglobulin$^{-/-}$NOD/SCID mice transplanted with human $CD34^+$ HPC expressing a constitutively active PKB mutant contain increased human BDCA-1^+ DC compared with mice transplanted with control HPC (52), indicating a supportive role for PKB in DC development. Although the PI3K/PKB axis has many downstream effectors, the impaired generation of DC following in vivo administration of rapamycin, a pharmacological inhibitor of mammalian target of rapamycin (mTOR), to mice (53) indicates that activity of PKB target mTOR complex 1 (mTORC1) is required. However, circulating $CD11c^+$ DC numbers were unaffected in kidney transplant patients treated with rapamycin (54).

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. Pharmacological inhibition of PI3K or mTOR inhibits in vitro GM-CSF-driven human DC development from monocytes (54-57) and hematopoietic progenitors (52, 56) due to reduced proliferation and survival of DC precursors. Increased signaling improves survival of DC and their precursors, and ectopic or GM-CSF-independent activation of this axis can even rescue these cells from GM-CSF-deprivation-induced apoptosis (52, 58-61). Although the survival of most DC lineages appears to be supported by PI3K-dependent signaling, important differences between distinct subsets are apparent. Human CD34-derived migratory DC are independent of PI3K and mTOR activity (52), but survival of human monocyte-derived DC requires activity of this pathway (56). Conversely, CD34-derived migratory pre-DC require PI3K and mTOR activity to survive, whereas monocytes are resistant to inhibition of PI3K or mTOR (52, 56). Contradictory experiments were shown in a recent study that reported apoptosis of monocytes two days after rapamycin administration (54). Rather than their being experimental inconsistencies, 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 and only one study has reported reduced CD1a expression (57). However, despite their DC phenotype, these cells are functionally

impaired (52, 54, 62-70), 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 monocyte-to-macrophage differentiation in monocyte-derived DC differentiation cultures (71-72). However, murine bone marrow-derived DC differentiation was independent of GSK-3β (73). Although PKB activity levels may affect phenotypic differentiation of some DC subsets, the greatest significance of this pathway lies in functional differentiation and promoting DC numbers by upholding sufficient expansion and survival.

MAPK

Few studies have critically evaluated the role of MAPK signaling during DC development. While 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, DC derived in vitro from murine ERK1$^{-/-}$ bone marrow progenitors were found to show increased surface expression of activation markers and enhanced T cell stimulation (74), 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. Pharmacological inhibition of MEK or ERK abrogates both differentiation and survival during human monocyte-derived DC development (57, 75). Although further direct evidence is limited, an association between loss of MEK/ERK activity and reduced DC development has been reported by several groups (76-78). Interestingly, activation of p38 MAPK by stimuli such as tumor-secreted factors, mycobacterial infection or LPS can impair GM-CSF-driven DC differentiation, whereas differentiation is improved by pharmacological inhibition of p38 MAPK (57, 79-81). Thus, GM-CSF seems to activate the MEK/ERK signaling module to promote DC development, while other factors may utilize 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

Members of the NF-κB/Rel transcription factor family are expressed at relatively high levels in DC (82). Individual knockout of the canonical NF-κB proteins RelA, c-Rel or p50 had no effect on the DC populations present in mouse spleen, but spleens of mice with a combined RelA and p50 deficiency contained reduced CD11c^{+} DC (83). Moreover, RelA$^{-/-}$p50$^{-/-}$ hematopoietic progenitors were unable to generate DC following adoptive transfer, and their DC differentiation ability in GM-CSF-driven cultures was strongly impaired (83). 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 (84-86). 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 processes contributing to DC development.

Pharmacological- or viral transduction-mediated NF-κB inhibition strongly reduces cell yields in DC differentiation cultures from human monocytes (85) and human or mouse hematopoietic progenitors (83, 85). The survival of both DC precursors (84-85) and differentiated DC (85-86) 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 (83), suggesting that differentiation was unaffected. However, canonical NF-κB inhibition in human monocytes and hematopoietic progenitors impaired their acquisition of a DC phenotype (85, 87), 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 pharmacological NF-κB inhibitors (85), whereas CD34-derived pre-Langerhans cells emerged despite NF-κB inhibition (85). However, terminal differentiation of pre-Langerhans cells was hindered by introduction of an IκBα super-repressor (87).
GM-CSF-induced canonical NF-κB activation thus appears crucial to ensure differentiation and survival of DC precursors. Interestingly, despite their incomplete DC phenotype, antigen uptake, activation-induced costimulatory molecule expression and allogeneic T cell stimulation by human monocyte-derived DC generated in the presence of NF-κB inhibitors was comparable to control monocyte-derived DC (85). However, their cytokine production abilities might be affected (85). Similarly, DC generated in vitro from $p50^{-/-}c\text{-}Rel^{-/-}$ murine bone marrow progenitors had a reduced ability to produce IL-12 (83). Continued NF-κB inhibition after differentiation could be responsible for impaired functionality, since DC activation induced by pathogenic stimulation, T cell signals or inflammatory cytokines other than GM-CSF, is strongly dependent on canonical NF-κB activity (88). Although more detailed investigation is required to allow definitive conclusions, it is highly likely 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 of the different GM-CSF-activated signaling pathways (Figure 4). 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 promoting expansion and survival of DC precursors rather than their differentiation, although its activity is required to generate DC with full functionality. Finally, activation of MEK/ERK and canonical NF-κB transcription factors is associated with differentiation and survival. However, although in these studies the distinct signaling cascades are often regarded as separate entities, this is mostly not the case since the different pathways share signaling proteins, upstream initiators of signaling and proteins regulating negative feedback (Table 1). Therefore, manipulation of one signaling module will most likely also affect others, meaning that observed effects on DC development may be accounted for by other pathways than the canonical pathway the targeted protein belongs to.

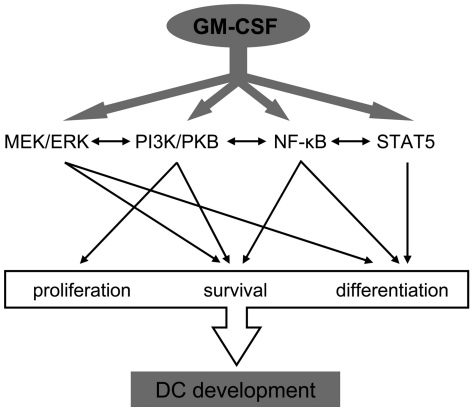

Figure 4. GM-CSF regulates DC development through an integrated molecular network
The signaling modules activated by GM-CSF form an integrated network with overlapping and separate functions that enable an adequate response to a wide range of situations. The currently known functions of the specific cascades in the regulation of GM-CSF-induced DC development are shown.

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

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, canonical NF-κB but not PI3K regulates phenotypic human monocyte- and CD34-derived myeloid DC differentiation (52, 56, 85) (Figure 4). This discrepancy is likely explained by PI3K-independent NF-κB activation. However, it seems prudent to keep inter-pathway regulation in mind when trying to assemble a reliable vision on the molecular aspects of GM-CSF-driven DC development.

Next to the functional segregation between the distinct signaling cascades with regard to processes such as DC subset differentiation, other processes appear to be broadly regulated. An example of a process supported by several GM-CSF-activated signaling modules is survival (Figure 4). Such broad regulation is helpful in ensuring survival under different circumstances, for example in situations in which environmental stimuli other than GM-CSF hinder survival-regulation through a specific signaling pathway. Furthermore, in a recent model of GM-CSF-induced hematopoiesis, low GM-CSF concentrations were suggested to activate only specific signaling modules, whereas additional intracellular signaling pathways were activated in the presence of high GM-CSF concentrations (30). Since survival could be

mediated through the signaling cascade activated by low GM-CSF concentrations, survival was ensured under all circumstances, while other processes such as proliferation were only initiated when the concentration of GM-CSF was high enough. 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 others activity. The combination of overlapping and separate functions of the distinct signaling proteins enables an adequate response to a wide range of situations.

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, 89), but how the intracellular signaling pathways regulating DC development modulate 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 (90), a factor shown to promote the commitment of human multipotent hematopoietic progenitors to myeloid progenitors generating DC (91). Additionally, the promoter of IRF4, a gene involved in subset-specific DC development, contains several putative NF-κB binding sites (92). RelA, c-Rel and p50 bind IRF4 promoter elements during human monocyte-derived DC differentiation (92). For murine DC, IRF4 expression was shown to be of particular relevance for GM-CSF-driven conventional DC differentiation in vitro, while spleens of $IRF4^{-/-}$ mice lack $CD8\alpha^-$ DC, but $CD8\alpha^+$ DC are unaffected and numbers of plasmacytoid DC are only modestly reduced (93-94). Another subset-specific regulator is RelB. Activation of this 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 (95-97). Similar to IRF4, RelB is crucial in the differentiation of $CD8\alpha^-$ splenic DC (98), and within the human immune system RelB was shown required for interstitial DC but not Langerhans cell differentiation (87). Besides affecting lineage decisions, canonical NF-κB-mediated regulation of RelB may contribute to the development of DC with a more activated phenotype, since this protein has been associated with DC immunogenicity (99). Finally, NF-κB is known as a major inducer of inflammatory cytokine and costimulatory molecule expression in terminally differentiated DC (88) and its activation by GM-CSF may therefore affect the functions of GM-CSF-differentiated DC.

Next 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 (37, 44). More direct may be its effect on PU.1 and C/EBPα (40, 100-101). Although these proteins are required during DC differentiation (91, 102), excessive expression of C/EBPα can compromise DC lineage commitment (91) and PU.1 expression levels have also been suggested to affect hematopoietic lineage choices (103-104). STAT5-mediated downregulation (40, 100-101) may be involved in maintaining the correct levels required for DC development. Besides, regulation of PU.1 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 high

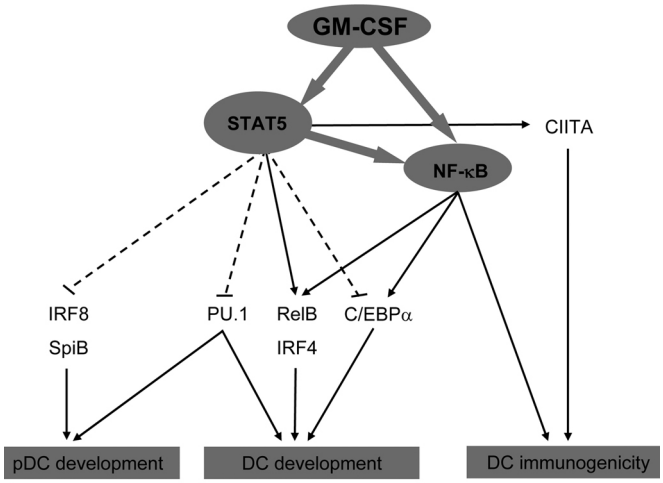

Figure 5. GM-CSF-induced regulation of the DC transcriptional program mediated through STAT5 and canonical NF-κB
STAT5 and canonical NF-κB transcription factors can directly regulate the DC transcriptional program. Regulation of RelB, IRF4, PU.1 and C/EBPα expression not only promotes DC differentiation in general, but may also influence subset distribution among the DC generated. The inhibitory actions of STAT5 towards IRF8 and SpiB explain the negative impact GM-CSF has on the plasmacytoid DC lineage, while effects on genes involved in DC functionality may promote the immunogenicity of DC differentiated with GM-CSF.

PU.1 expression levels at the cost of interstitial DC development (105). In addition, murine $CD8\alpha^-$ but not $CD8\alpha^+$ splenic DC are dependent on PU.1 for their development (106). Other functions by which STAT5 may regulate subset distribution include the inhibition of IRF8 and SpiB gene transcription (37). These factors are mainly associated with plasmayctoid DC development (2, 89), and the inhibitory actions of STAT5 towards these three genes could explain the negative impact GM-CSF-activated STAT5 has on the plasmacytoid DC lineage. Indeed, spleens of mice deficient for IRF8 show specific reductions in plasmacytoid DC and $CD8\alpha^+$ DC (94). However, human subjects with IRF8 mutations resulting in strongly reduced IRF8 activity show general loss of DC in peripheral blood (107), 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) (43), which is essential for transcriptional activity of the MHC class II promoter. Since STAT5 also stimulates DNA binding and transcriptional activity of NF-κB (108), GM-CSF-induced STAT5 activation may increase the intrinsic immunogenicity of DC 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 activation and transcriptional activity (109-110), 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 (111). In addition, direct inhibitory effects of ERK on CIITA expression have been proposed (112). 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, IRF8, SpiB, PU.1 and C/EBPα, 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 DC.

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). This module generally directs proliferation through transcriptional and translational control (113-115), but the specific mechanisms applied during GM-CSF-induced DC development are undetermined. Although direct evidence is currently lacking, considering their important role in other hematopoietic lineages it appears highly likely that MEK/ERK and JAK2/STAT5 are also involved in DC

precursor expansion (110, 116). Knowledge on the molecular regulation of DC survival is little more available. The IKK complex inhibits pro-apoptotic Forkhead box O (FOXO) transcription factors (117) and activates canonical NF-κB transcription factors that induce the expression of Bfl-1/A1, Bcl-2, Bcl-XL and IAP, proteins involved in anti-apoptosis (118-121). Similarly, MEK/ERK signaling has been shown to regulate the expression of BAD, Bcl-2 and Bcl-XL (26, 116), 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 (110, 122). Although Bcl-2, FOXO1 and particularly Bcl-XL (83) have been associated with DC survival, direct evidence that the GM-CSF-activated signaling modules indeed regulate DC survival through manipulation of these factors is currently not available. In contrast, GM-CSF-induced mTORC1-mediated survival of human monocyte-derived DC has been shown to depend on regulation of anti-apoptotic Mcl-1 (75). In addition, GSK-3β activity, which is inhibited by phosphorylation by PKB, can induce apoptosis by Mcl-1 destabilization (123). Furthermore, PI3K, PKB and mTORC1 regulate many other proteins, including FOXO1, BAD and Bcl-2 that could contribute to the maintenance of DC (109). The above mentioned factors involved in DC survival regulation were mainly identified for differentiated DC. Although tempting, extrapolation of these findings to DC precursors may be inappropriate due to the differential expression of apoptosis regulators at distinct DC differentiation stages (52). In addition, subset-specific apoptosis regulators as well as signal transduction pathways regulating survival have been reported (52, 124), stressing the need to separately evaluate each DC subset. Overall, the molecular control of DC viability and expansion in general, and of 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 comparison of GM-CSF- and Flt3L-driven DC development

GM-CSF and Flt3L are both key cytokines in DC development, but the DC they generate show specific characteristics that can be explained by the molecular program activated by either cytokine. Both GM-CSF and Flt3L activate STAT3, PI3K/PKB and MEK/ERK signaling. Variations in the magnitude of activation of each of these modules and/or in their relative activity compared to each other may account for the differential DC expansion induced by injection of GM-CSF and Flt3L (2), but alternative explanations also exist. Major differences between GM-CSF- and Flt3L-induced signal transduction include the activation of STAT5 and the direct activation of canonical NF-κB by GM-CSF, events that are absent in Flt3L-stimulated cells. Whereas Flt3L induces DC commitment and differentiation through activation of STAT3 (38, 41), thereby supporting the development of all DC subsets (5), a different DC subset-distribution can be found in GM-CSF-dependent cultures due to the activities of STAT5 and canonical NF-κB (Figure 5). However, although the actions of GM-CSF inhibit plasmacytoid DC development in vitro (19), plasmacytoid DC numbers were unchanged in GM-CSF$^{-/-}$ mice (16), indicating that, at least under steady state conditions, the in vivo effects of GM-CSF on plasmacytoid DC development may be limited.

Under inflammatory conditions, GM-CSF secretion is augmented (21) and its role becomes more apparent. Besides supporting the differentiation of both conventional and unconventional DC precursors such as monocytes, thereby not only increasing the rate of DC development but also changing the composition of the DC pool (4, 24), GM-CSF increases the intrinsic immunogenicity of the DC generated (Figures 5 and 6). Flt3L-activated STAT3 inhibits canonical NF-κB activity as well as the expression of MHC class II proteins and costimulatory molecules (125-128), and promotes transcription of IDO (129), actions that account for the tolerogenic function of DC generated in response to Flt3L (130) (Figure 6). In contrast, through activation of STAT5 and canonical NF-κB transcription factors, GM-CSF

promotes the development of immunogenic DC (Figures 5 and 6). Thus, steady state Flt3L-induced DC development to maintain homeostasis is induced through STAT3, yielding relatively tolerogenic DC of all subsets. Increased GM-CSF production due to inflammation initiates a more immunogenic state with STAT5- and NF-κB-dependent DC differentiation, leading to the generation of specific DC subsets with enhanced intrinsic immunogenicity.

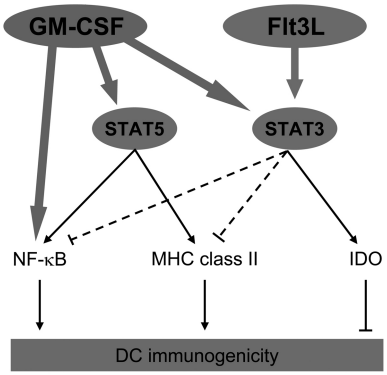

Figure 6. A molecular explanation for the reciprocal effects of GM-CSF and Flt3L on DC immunogenicity
The complementary effects GM-CSF and Flt3L have on the DC they generate can be explained by their molecular actions. Through activation of STAT3, Flt3L promotes expression of IDO but inhibits canonical 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 transcription, STAT5 overrules the tolerogenic function of STAT3, resulting in the development of highly immunogenic DC.

Clinical implementation

The key role GM-CSF has during the generation of DC, in particular those with an immunogenic phenotype and functionality, underlines its importance, but in some cases also contributes to diseases caused by dysregulated immune activation. In mice susceptible to severe experimental autoimmune encephalomyelitis, T cell-derived GM-CSF contributed to inflammation of the central nervous system by the recruitment and activation of myeloid antigen-presenting cells (131-132). Similarly, the development of sterile acute inflammatory arthritis and peritonitis was shown to depend on GM-CSF-induced inflammatory monocyte-derived DC (133). Conversely, examples of the detrimental effects of inefficient GM-CSF-regulated immunity are also available (134-135). The recognition of GM-CSF as an important regulator of hematopoiesis in general and as an inducer of immunogenic DC development in particular has issued its inclusion in the treatment of several diseases.
By reducing febrile neutropenia after cytotoxic chemotherapy, recombinant GM-CSF significantly contributes to the supportive care of cancer patients (136). Furthermore, GM-CSF has been successfully included in mobilization regimens for hematopoietic stem cell transplantation (HSCT). Administration of GM-CSF induces three to ten times higher DC yields during autologous stem cell mobilization, leading to rapid engraftment with reduced mucositis and fewer infections, without an increase in graft-versus-host disease (137). A recent pilot-study described that GM-CSF administration during both mobilization and engraftment of stem cells improved the reconstitution of DC after autologous HSCT (138). In contrast, DC numbers were decreased in allogeneic grafts, with a more pronounced reduction in plasmacytoid DC (138).
Besides aiding immune reconstitution, GM-CSF-induced DC have been applied to induce anti-tumor immunity. In early clinical studies, administration of GM-CSF has been shown to cause anti-tumor immune responses as well as tumor regression, although complete remission was rare (139). Several strategies have tried to increase the effectiveness of GM-CSF administration by direct injection into the tumor or co-injection of GM-CSF and tumor antigens (139). Furthermore, resected tumors were processed ex vivo, infected with

viruses expressing human GM-CSF, irradiated and finally injected into the patient in the hope that GM-CSF would attract DC and promote an immunogenic response after capture of the tumor antigens (140-141). Finally, therapeutic vaccination strategies based on the ex vivo generation and tumor antigen-loading of DC mostly use GM-CSF-based protocols to generate DC (142). The results of initial trials following either of these strategies are promising. Importantly, although Flt3L administration has also been suggested to support DC-mediated tumor rejection, vaccination with irradiated GM-CSF-secreting tumor cells stimulated more potent anti-tumor immunity than vaccination with tumor cells secreting Flt3L (143).

Besides its potential use in promoting immunogenic tumor rejection, GM-CSF is used as potent adjuvant in several vaccination programs. Codelivery of hepatitis B antigens with GM-CSF elicited high antibody titers and T cell responses in normally nonresponsive mouse strains (144), and administration of GM-CSF together with the standard hepatitis B virus vaccine significantly increased the response rates in patient groups that are known for their immunization difficulties (145). Beneficial effects have also been reported in vaccination strategies for the treatment of hepatitis C virus and human immunodeficiency virus, and of fungal or mycobacterial infections.

Although several methods have been developed to increase the effectiveness of GM-CSF while at the same time attempting to limit its activity to the intended purpose, several problems remain. Administration of GM-CSF can have widespread side effects including fever, headaches and flu-like symptoms (146), and as a consequence the maximum dose that can be applied is limited. In addition, the benefit of GM-CSF can be counteracted by the presence of anti-inflammatory signals, for example those present in the tumor micro-environment. Furthermore, simply adding GM-CSF is not always effective at increasing signaling due to negative feedback mechanisms. Finally, administration of GM-CSF increases signaling along all GM-CSF-activated pathways, and it is therefore not possible to distinguish between the different GM-CSF-induced processes.

To circumvent the drawbacks of using whole GM-CSF, targeting the signaling proteins that regulate GM-CSF-driven effects on DC could be attempted. The current knowledge on the molecular regulation of GM-CSF-induced DC development reveals several candidates for these purposes, including signaling proteins involved in the regulation of DC numbers, subset distribution, the lifespan and the intrinsic immunogenicity of DC. The supporting role of the PI3K-PKB-mTOR signaling module in expanding DC progenitors (52) suggests its exploitation to boost DC yields. Based on the decisive function STAT5 has in DC lineage commitment (36-37, 40), it appears a candidate protein to manipulate DC subset-specification. Alternatively, the STAT5-regulated factors that are responsible for its effects on lineage decisions, such as PU.1 or IRF8, could be targeted. However, whereas in mice STAT5-mediated plasmacytoid DC inhibition is mediated through IRF8 (37), IRF8 loss-of-function in humans results in a more widespread DC deficiency (107), indicating that this might not be a good target in humans. RelB appears to have a subset-restricted function in both humans and mice (87, 147), and targeting RelB could provide a potential strategy to specifically regulate human interstitial DC development.

Besides manipulating subset development, the contribution of a particular subset could be supported through regulation of its survival or activation. Mouse bone marrow-derived DC and human monocyte-derived DC expressing constitutively active PKB are highly activated DC with improved longevity that are able to eradicate tumors more efficiently than control DC (61). Similarly, siRNA-mediated deletion of the cell-intrinsic PI3K negative regulator PTEN in murine bone marrow-derived DC improved their survival, maturation, expression of CCR7 and T cell activation, resulting in increased anti-tumor immunity (59). Other potential proteins that could be targeted to manipulate DC immunogenicity include STAT3,

STAT5, canonical NF-κB proteins, mTOR and RelB. As described above, GM-CSF-induced activation of STAT5, NF-κB and mTOR during or after DC differentiation is associated with the development of immunogenic DC, and artificial activation of these proteins could thus support DC functionality. Due to its tolerogenic effect, decreasing STAT3 activity could also contribute to the development of immunogenic immune responses. In mice, STAT3 depletion considerably enhanced DC function and anti-tumor immune responses (148-149). In contrast, silencing of RelB resulted in DC inducing tolerance to transplanted hearts and treating autoimmune myasthenia gravis (150-152).
Although these results are promising, direct targeting of GM-CSF-regulated signaling proteins does not completely resolve the side effects of GM-CSF administration. Similar to the GM-CSFR, signaling proteins are widely expressed and perform important regulatory functions in various cell types, demanding specific targeting to the intended cells. In humans, targeting to early progenitors is currently not possible in vivo due to the missing definition of DC-specific progenitors. Alternatively, ex vivo manipulation and/or targeting of further differentiated DC precursors could be attempted. Additional remaining problems are environmental signals and/or negative feedback that alter the intended signaling. Finally, although targeting of specific signaling proteins theoretically enables manipulation of selected processes such as differentiation or proliferation, consequences on other proteins and processes due to the interrelation of the different pathways can not be avoided completely. However, 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 DC, and will hopefully lead to novel strategies to manipulate DC biology for therapeutic purposes.

Concluding remarks

Over the last decade, there has been considerable interest in the cytokines and cytokine-induced molecular events regulating DC development, and despite its initial neglect, understanding of GM-CSF-driven DC differentiation is now regarded highly relevant. The four 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 (Figure 4). 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 under a wide range of circumstances. However, the realization that the different signaling modules are in fact interconnected stresses the need to carefully evaluate the available data, since effects on DC development observed after manipulation of a specific signaling protein may be caused by another pathway than the canonical pathway the targeted protein belongs to. This is a subject that should be given more attention in the future.
The molecular actions of GM-CSF explain the characteristics specific for GM-CSF-differentiated DC compared to, for example, DC differentiated in response to Flt3L. GM-CSF-specific activation of STAT5 and canonical NF-κB proteins not only affects the subset distribution of developing DC, but also promotes development of DC with a relatively immunogenic phenotype and functionality (Figures 5 and 6). Although the identification of the signaling proteins and transcription factors responsible for GM-CSF-induced characteristics could be of use in the improvement and/or development of new therapies using DC, several issues remain to be resolved before this knowledge can be applied fully. In particular, further understanding of the ontogenetic background of DC and of their contribution to the development of immune responses under different pathophysiological situations is required.

Besides further elucidation of the signaling pathways regulating DC development, future research should be directed at clarification of these issues to enable the use of DC with tightly controlled functional abilities in novel therapeutic applications.

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3

HUMAN CD34-DERIVED MYELOID DENDRITIC CELL DEVELOPMENT REQUIRES INTACT PI3K-PKB-MTOR SIGNALING

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Abstract

Dendritic cells (DC) are comprised of different subsets which exhibit distinct functionality in the induction and regulation of immune responses. The myeloid DC subsets, including interstitial DC (intDC) and Langerhans cells (LC), develop from $CD34^+$ hematopoietic progenitors via direct DC precursors or monocytes. The molecular mechanisms regulating DC development are still largely unknown and mostly studied in mice. Phosphatidylinositol 3-kinase (PI3K) regulates multiple processes in myeloid cells. The present study investigated the role of PI3K signaling in the development of human CD34-derived myeloid DC. Pharmacological inhibition of PI3K or one of its downstream targets mTOR reduced intDC and LC numbers in vitro. Increased activity of this signaling module by introduction of constitutively active protein kinase B (PKB/c-AKT) increased the yields of human DC precursors in vitro as well as in transplanted β2-microglobulin$^{-/-}$NOD/SCID mice in vivo. Signaling inhibition during differentiation did not affect the acquisition of a DC phenotype, while proliferation and survival strongly depended on intact PI3K-PKB-mTOR signaling. Interestingly however, this pathway became redundant for survival regulation upon terminal differentiation, which was associated with an altered expression of apoptosis regulating genes. Although dispensable for costimulatory molecule expression, the PI3K-PKB-mTOR signaling module was required for other important processes associated with DC function, including antigen uptake, LPS-induced cytokine secretion, CCR7 expression and T cell stimulation. Thus, PI3K-PKB-mTOR signaling plays a crucial role in the development of functional CD34-derived myeloid DC. These findings could be used as a strategy to manipulate DC subset distribution and function in order to regulate immunity.

Introduction

Dendritic cells (DC) are professional antigen (Ag) presenting cells that play a crucial role in immunity, ranging from tolerogenic to immunogenic responses (1). The type of the immune response is determined by the DC maturation state, the lifespan of the Ag-bearing DC, and the DC subtype (2-5). Based on surface markers, localization, functional abilities and ontogeny, a large variety of DC subsets can be recognized. The myeloid migratory DC subtypes, including Langerhans cells (LC) and interstitial DC (intDC), are known for their superior T cell priming ability compared with for example plasmacytoid DC.

DC develop from $CD34^+$ hematopoietic progenitor cells (HPC) via subset specific precursors. These are present either as direct precursor DC (pre-DC) or as immune effector cells with their own function in immunity, such as monocytes, giving rise to DC when necessary (6-7). Whereas the cytokines responsible for the differentiation and survival of DC are becoming increasingly clear (7), the molecular mechanisms regulating DC development remain poorly understood. Furthermore, the majority of studies examining the role of specific signaling pathways and transcription factors have focused only on DC development in mice (7).

A likely candidate for the regulation of DC development is phosphatidylinositol 3-kinase (PI3K), which regulates multiple cellular processes in a variety of hematopoietic cells, including erythrocytes, neutrophils, eosinophils and T and B lymphocytes (8-10). Signaling downstream of activated PI3K includes the activation of protein kinase B (PKB, also called c-AKT) and regulation of several downstream substrates such as mammalian target of rapamycin (mTOR), glycogen synthase kinase-3β and Forkhead box O transcription factors (10-11). In myeloid DC, PI3K signaling has been shown to be involved in the regulation of phenotypic and functional maturation as well as activation-induced survival (12-16). However, the importance of PI3K and its downstream substrates for human myeloid DC development is still largely undefined and the few available studies showing effects on differentiation or yields are mainly limited to monocyte-derived DC (mo-DC) (17-19).

DC differentiation from monocytes is the most widely utilized model to study DC biology (20). To investigate the development of myeloid DC subsets from a less committed progenitor, $CD34^+$ HPC can be used. Upon culture with GM-CSF, TNF-α and SCF, the two functionally different myeloid subsets, intDC and LC, develop via independent pathways and can therefore be separately studied (21-22). The differentiation in two phases, with pre-DC developing prior to terminal differentiation, enables detailed analysis of CD34-derived myeloid DC development by this in vitro system. In addition, CD34-derived DC development can also be studied in vivo, by xeno-transplantation of human HPC into immune-deficient mice (23-25).

In the present study, we investigated the importance of PI3K signaling for the development of functional intDC and LC from human cord blood $CD34^+$ HPC. PI3K-induced mTOR activation, mediated by PKB, was shown to be crucial for human myeloid CD34-derived DC development in vitro and in vivo. Precursor proliferation and survival strongly depended on this signaling module. Although inhibition of this pathway did not affect the acquisition of a DC phenotype, DC generated under PI3K or mTOR inhibition were functionally impaired. In contrast to the requirement for PI3K signaling during development, the survival of terminally differentiated CD34-derived myeloid DC was not dependent on PI3K or mTOR activity. However, this signaling module remained required for other important processes associated with DC function. These findings demonstrate the importance of the PI3K-PKB-mTOR signaling module during CD34-derived myeloid DC development, providing possibilities to manipulate DC subset distribution and function to regulate immunity.

Materials and Methods

Generation of CD34-derived myeloid dendritic cells
Umbilical cord blood samples were obtained ex-uterine according to legal guidelines. $CD34^+$ hematopoietic progenitor cells were isolated and cultured as described previously (22, 26). In brief, $CD34^+$ cells were isolated from mononuclear fractions through positive selection using anti-CD34-coated microbeads and MS separation columns (both Miltenyi Biotec GmBH, Bergish Gladbach, Germany) to a purity of 85-98%. After cryopreservation to standardize differentiation, cells were cultured in complete medium containing RPMI-1640 (Invitrogen, Breda, The Netherlands) supplemented with 8% heat inactivated fetal calf serum (FCS; Hyclone, Thermo Fisher Scientific, Etten-Leur, The Netherlands), 10 mM Hepes (Invitrogen), 2 mM L-glutamine (Invitrogen), 50 μM β-mercaptoethanol (Merck, Darmstadt, Germany) and penicillin/streptomycin (Invitrogen). From day 0-6, the cells were cultured in complete medium supplemented with 100 ng/ml GM-CSF (Schering-Plough, Houten, The Netherlands), 25 ng/ml SCF (PeproTech, London, UK), 2.5 ng/ml TNF-α (R&D systems, Abingdon, UK) and 5% heat inactivated AB^+ pooled human serum (4 different donors, Sanquin, Rotterdam, The Netherlands). Then, cells were harvested, washed and further cultured in complete medium containing only GM-CSF (100 ng/ml). Where indicated, LY294002 (LY; 2 μM unless indicated otherwise; Biomol, Plymouth Meeting, PA) or Rapamycin (Rapa; 20 nM unless indicated otherwise; Biomol) was added to the cultures.

Activation of DC
Terminally differentiated DC were activated by addition of LPS (100 ng/ml; Invivogen, San Diego, CA). Alternatively, cells were co-cultured with CD40L-transfected L cells (L-CD40L) (27) in a DC:L cell ratio of 4:1. Non-transfected L cells (L-Orient) served as control cells. During activation, cells were incubated in the presence of 100 ng/ml GM-CSF.

Viral transduction of $CD34^+$ cells
A bicistronic retroviral DNA construct, co-expressing the genes encoding myrPKB and eGFP, with an internal ribosomal entry side (IRES) preceding eGFP (LZRS-myrPKB-IRES-eGFP; kindly provided by Dr H. Spits, Amsterdam, The Netherlands) was used to transduce $CD34^+$ cells. A second vector expressing only IRES followed by eGFP (LZRS-IRES-eGFP) was used as negative control. The retroviral packaging cell line Phoenix-ampho (28) was stably transfected with 10 μg DNA by calcium phosphate coprecipitation. 24 hours before transfection, cells were plated into 6 cm dishes. DMEM medium (Invitrogen) was refreshed 16 hours after transfection, and after an additional 24 hours, cells were cultured in selection medium containing 10 μg/ml puromycin (Sigma) until confluent. Selection medium was then replaced by $CD34^+$ culture medium. After 24 hours, viral supernatants were collected, filtered through an 0.2 μm filter and subsequently frozen.
$CD34^+$ progenitors were transduced in 24-well culture dishes precoated with 10 $\mu g/cm^2$ recombinant human fibronectin fragment CH-296 (RetroNectin, Takara, Otsu, Japan) for 16 hours. Transduction was performed by adding 0.5 ml viral supernatant to 0.5 ml medium containing 5-30 x 10^4 cells. After 24 hours, 0.7 ml medium was removed and 0.5 ml fresh viral supernatant was added together with 0.5 ml fresh medium. For in vitro experiments, cells were transduced in complete RPMI medium supplemented with GM-CSF, TNF-α, SCF and human serum in the above described concentrations. $CD34^+$ progenitors used for in vivo experiments were transduced in IMDM medium (Invitrogen) supplemented with 8% heat inactivated FCS, penicillin/streptomycin, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml Flt3L (Peprotech) and 50 ng/ml SCF.

Cell surface phenotype analysis and apoptosis detection by flow cytometry
For phenotypic analysis, cells were washed in PBS containing 1% bovine serum albumin (BSA), 1% heat inactivated human serum and 0.02% NaN_3. Labeling of cell surface markers was performed on ice, using fluorochrome-conjugated antibodies against the following Ag: CD1a (HI149, BD Biosciences, Breda, The Netherlands), CD14 (MΦP9, BD Biosciences), Langerin/CD207 (DCGM4, Beckman Coulter, Woerden, The Netherlands), CD1c (AD5-8E7, Miltenyi Biotec, Utrecht, The Netherlands), CD20 (L27, BD Biosciences), CD86 (Fun-1, BD Biosciences), CD83 (HB15e, BD Biosciences), HLA-DR (L243, BD Biosciences) and CCR7 (150503, R&D systems). Apoptosis was detected by determination of phosphatidyl serine exposure and membrane permeability. Cells were harvested, washed in Annexin buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), incubated with Annexin V-FITC

(BD Biosciences) for 30 minutes on ice and subsequently taken up in 1 µg/ml Propidium Iodide (PI; Sigma-Aldrich). Transduced cells were incubated with Annexin V-PE and 7-AAD (both BD Biosciences) in Annexin buffer for 30 minutes. Assessment was performed using a FACSCalibur or FACSCanto II (BD Biosciences) and data were analyzed using Cell Quest Pro and FACSDiva software.

Cell cycle progression analysis
Cells were harvested, washed in PBS and taken up in PBS/5mM EDTA. Ethanol was added and cells were fixed in 50% ethanol for 30 minutes on ice. Then, cells were washed and subsequently incubated with PBS/5mM EDTA with 40 µg/ml RNAse A (Promega, Madison, WI) for 30 minutes at room temperature. PI (50 µg/ml) was added and fluorescence intensity was immediately measured by flow cytometry (FACSCalibur).

Isolation of subset-specific precursors or transduced cells by FACSorting
Subset-specific precursors or retrovirally transduced cells were sorted using a FACSAria (BD Biosciences). Control cultures were harvested at day 6, incubated with fluorochrome-conjugated antibodies against CD1a and CD14, life gated and sorted into $CD14^{+}CD1a^{-}$ and $CD14^{-}CD1a^{+}$ fractions. $eGFP^{+}$ cells were isolated 3-4 days after transduction.

Western blot
Cells were washed in PBS, lysed in Laemmli sample buffer (0.12 M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 µg/µl bromophenol blue and 35 mM β-mercaptoethanol) and boiled for 5 minutes. Protein concentrations were determined by Lowry method. Equal amounts of total lysate (10 µg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to a PVDF membrane (Millipore, Bedford, MA) and incubated with blocking buffer (Tris buffered saline/Tween20) containing 5% BSA or low fat milk before probing with antibodies against human phosphorylated S6 (rabbit polyclonal, Cell Signaling Technology, Danvers, MA) and tubulin (mouse monoclonal, Sigma-Aldrich). Subsequently, blots were incubated with HRP-conjugated secondary antibodies (Dako, Glostrup, Denmark). Enhanced chemical luminescence was used as a detection method according to the manufacturer's protocol (Amersham Pharmacia, Amersham, United Kingdom).

Reverse Transcription-Multiplex Ligation-dependent Probe Amplification (RT-MLPA)
Total RNA was isolated using the RNAeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Optical density (OD)260/280 ratios were measured to determine the quantity and purity of RNA preparations and RNA integrity was checked using the Agilent RNA 6000 nano assay kit according to manufacturer's instructions (Agilent Technologies, Santa Clara, CA). RT-MLPA was performed as described before (29). In brief, RNA samples (40-60 ng of total RNA) were reverse transcribed using a gene-specific probe mix. The resulting cDNA was annealed to multiplex ligation-dependent amplification probes that had been prepared from two oligonucleotides, a short synthetic oligonucleotide (Biolegio, Malden, The Netherlands) and a phage M13-derived long probe oligonucleotide. The two oligonucleotides annealed to adjacent sites on a target sequence and were subsequently ligated by Ligase-65 (MRC-Holland, Amsterdam, The Netherlands). Ligation products were amplified by PCR, each ligated probe giving rise to an amplification product of unique length. The resulting DNA mixture was analyzed by capillary sequencer and standard software for identification and relative quantification of the PCR products. Analyzed data were exported to Microsoft Excel spreadsheet software for further analysis. To normalize for variations in total signal between samples, the sum of all peak data was set at 100% for each sample. Individual peaks were calculated relative to the 100% value. Signals below the detection limit in medium were assigned a value corresponding to the threshold value.

Transplantation of β2-microglobulin$^{-/-}$NOD/SCID mice with human $CD34^{+}$ progenitors
Protocols for mouse experiments were approved by the local animal experimental committee. The β2-microglobulin$^{-/-}$ nonobese diabetic/severe combined immune deficient (NOD/SCID) mice were bred and maintained under sterile conditions in microisolator cages and provided with autoclaved food and acidified water containing 111 mg/l ciprofloxacin (Ciproxin). 8- to 10-week-old mice, sublethally irradiated with 250 cGy x-rays, received transplants via tail vein injections with approximately 0.5 x 10^{6} unsorted retrovirally transduced cord blood-derived $CD34^{+}$ hematopoietic progenitors along with 10^{6} irradiated (1500 cGy) $CD34^{+}$-depleted cord blood-derived accessory cells. Six weeks after transplantation, the mice were sacrificed and both tibiae and femora were flushed. Bone marrow cells were analyzed for the

presence of human myeloid DC using flow cytometry.

Ag uptake assays
Ag uptake was analyzed as described before (30). In short, fluid phase and lectin-mediated endocytosis were measured as the cellular uptake of 100 µg/ml Lucifer Yellow dipotassium salt (Molecular Probes, Invitrogen) and 100 µg/ml dextran-FITC (Dextran^{FITC}, 40,000 MW, Molecular Probes, Invitrogen) respectively. Approximately 5 x 10^4 DC were incubated for 2 hours at 37^{o}C in the presence of culture medium containing Lucifer Yellow or Dextran^{FITC}. Negative controls were incubated with the respective Ag at 4^{o}C. Staining was evaluated by flow cytometry and Ag uptake was calculated by subtracting staining at 4^{o}C from staining at 37^{o}C.

Detection of cytokine production
Cytokine production was measured in supernatants of stimulated cells by multiplex particle based flow cytometry or ELISA. Concentrations of IL-6, IL-10 and TNF-α were measured with the Bio-Plex system using Bio-Plex Manager software version 4.0 (Bio-Rad Laboratories, Hercules CA), which uses Luminex xMAP technology as previously described (31-32). The multiplex data (cytokine concentrations) were digitized to create a cytokine portrait enabling the complete spectrum of cytokines to be visualized. The detection limit of this procedure was 1.2 pg/ml. The commercially available ELISA kits for human IL-5 (eBioscience, San Diego, CA), IL-6 (eBioscience), IL-8 (Biosource, Invitrogen), IL-10 (eBioscience), IL-12p70 (eBioscience), IP-10 (Biosource, Invitrogen) and IFN-γ (eBioscience) were used according to manufacturer's instructions. The detection limits of these assays were 2 pg/ml (IL-6, IL-10 and IP-10), 4 pg/ml (IL-5, IL-12p70 and IFN-γ) and 5 pg/ml (IL-8).

Allogeneic mixed lymphocyte reaction (MLR)
Responder T cells were isolated from a buffy coat. The mononuclear fraction was incubated with anti-CD15- and anti-CD235-coated microbeads (Miltenyi Biotech GmBH) and PE-labeled antibodies against CD1c (AD5-8E7, Miltenyi Biotech GmBH), CD14, CD19 (J4.119, Beckman Coulter, Woerden, The Netherlands), CD56 (MY31, BD Biosciences) and CD123 (SSDCLY107D2, Beckman Coulter), followed by incubation with anti-PE-coated microbeads (Miltenyi Biotech GmBH). T cells were isolated through negative selection according to manufacturer's instructions (Miltenyi Biotech GmBH). Irradiated DC (30 Gy) were added in graded doses to 2 x 10^4 allogeneic T cells in 96-well round bottom plates in RPMI-1640 containing 8% heat inactivated FCS. Proliferation was quantified by incubation with 1 µCi (37 kBq) [methyl-^{3}H]thymidine (NEN™ Life Science Products, Inc., Boston, MA) during the last 18 hours of 6-day cultures.

Results

Inhibition of PI3K or mTOR strongly reduces CD34-derived myeloid DC yield
The importance of PI3K signaling for the development of CD34-derived myeloid DC was investigated by addition of a specific pharmacological PI3K inhibitor, LY294002 (LY), during DC development in vitro. Continuous exposure to LY from day 0 did not affect differentiation, as shown by the presence of CD14$^+$CD1a$^-$ pre-intDC and CD14$^-$CD1a$^+$ pre-LC at day 6 and 90% CD14$^-$CD1a$^+$ myeloid DC present at day 13 irrespective of the presence of PI3K inhibition (Figure 1A). Furthermore, the specific myeloid DC marker CD1c was expressed by the majority of the CD1a$^+$ cells in control as well as inhibitor cultures (data not shown). In addition, stimulation of these cells with LPS in the absence of LY induced comparable expression of CD83, CD86 and HLA-DR for all cells (Figure 1B). Thus, blockade of PI3K during DC development did not significantly affect the phenotype of DC. However, a strong reduction (64.4±7.9%) in the yield of CD1a$^+$ DC was observed at day 13 (Figure 1C).
To determine the mechanism by which PI3K regulates DC development, the role of mTOR, one of the proteins whose activity is regulated by PI3K signaling, was examined. Both LY and rapamycin (Rapa), a specific pharmacological inhibitor of mTOR, reduced the phosphorylation of S6 (Figure 1D), a target downstream of mTOR (33). Similar to LY, addition

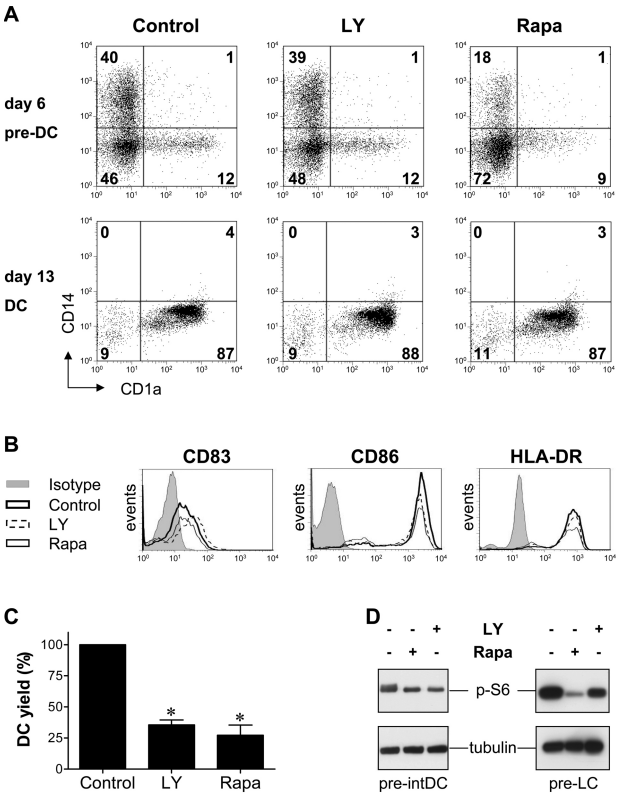

Figure 1. PI3K and mTOR are required for CD34-derived myeloid DC development, but do not regulate differentiation

LY, Rapa or their solvent DMSO was added to CD34-derived myeloid DC differentiation cultures at days 0, 4, 6 and 10. At day 13, cells were thoroughly washed and subsequently stimulated with LPS. ***A)*** *Day 6 (n=9) and day 13 (n=4) cells were harvested and analyzed for the expression of CD14 and CD1a. Viable cells were gated on FSC/SSC. Representative FACS plots are shown.* ***B)*** *LPS-stimulated cells were harvested at day 15. Cells were analyzed for the expression of CD83, CD86 and HLA-DR. Viable cells were gated on FSC/SSC. Representative FACS plots (n=2) are shown.* ***C)*** *At day 13, total cell numbers were determined by counting with trypan blue exclusion and absolute $CD1a^+$ DC numbers (including both $CD14^+$ and $CD14^-$ cells) were calculated and standardized to control cultures. Data shown represent mean±SEM $CD1a^+$ DC numbers of 4 independent experiments with different donors.* ***D)*** *LY, Rapa or DMSO was added to control day 6 FACSorted pre-DC. Cells were harvested after 2 days and whole cell lysates were prepared. Phosphorylation of S6 (p-S6) and tubulin expression were determined. * $p<0.05$, paired Student t-test on original data*

of Rapa allowed development of pre-DC as well as terminally differentiated DC from $CD34^+$ progenitors but significantly reduced the DC yield (Figure 1A,C). Also as observed for LY, LPS-induced costimulatory molecule expression was unaffected for these cells (Figure 1B). Since LY and Rapa strongly reduced DC numbers without affecting their phenotype, we conclude that both PI3K and mTOR activity are required for CD34-derived myeloid DC development, but these proteins seem to regulate other processes than differentiation.

PI3K-mediated regulation of pre-DC numbers by a combined effect on survival and proliferation

To investigate how PI3K and mTOR regulate DC development, we first focused on pre-DC development (day 0-6) and examined the consequences of PI3K and mTOR inhibition in more detail. Depending on the donor, control cultures resulted in a 3-50 fold increased cell yield after 6 days compared to day 0. Addition of either LY or Rapa significantly reduced cell yields in a dose-dependent manner (Figure 2A,B). At a concentration of 2 μM LY or 20 nM Rapa, which are both within the range of concentrations used in literature, cell growth was inhibited with respectively 67±9.7% and 83±12.5% (Figure 2A). Increasing the concentration of Rapa hardly induced additional effects (Figure 2B). Increased concentrations of LY, however, appeared to be extremely toxic (Figure 2B), which might be explained by other mechanisms than mTOR inhibition (34). These data show that the expansion of DC precursors requires both PI3K and mTOR activation.

To determine whether the reduced expansion in PI3K- or mTOR-inhibited cultures was due to decreased cell survival and/or proliferation, cell viability and cell cycle profiles were analyzed at different time points during the 6-day culture period. As demonstrated by increased

Annexin V binding in both the PI$^-$ and PI$^+$ cell population upon culture in the presence of LY or Rapa, PI3K and mTOR inhibition induced apoptosis at all time points (Figure 2C,D). In addition, a decreased cell cycle progression was observed, as shown by the increased proportion of cells in G0/1 (Figure 2E,F). These data show that a combination of reduced survival and decreased proliferation was responsible for the reduction in pre-DC yields found upon inhibition of PI3K or mTOR activity.

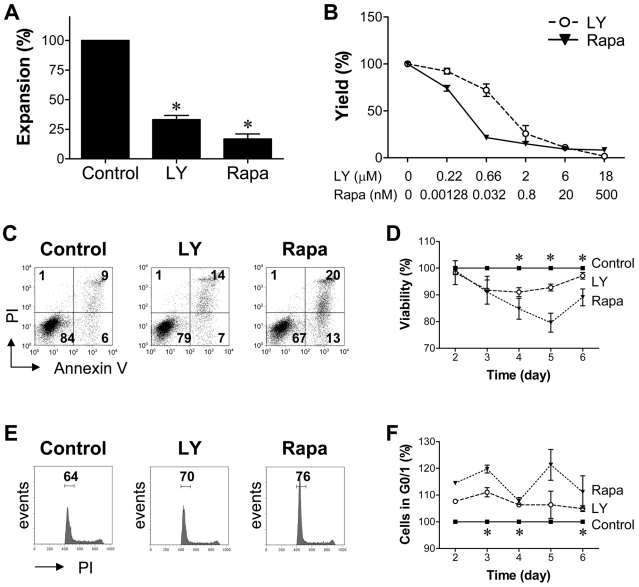

Figure 2. Inhibition of PI3K or mTOR reduces survival and proliferation during pre-DC development

LY, Rapa or DMSO was added at days 0 and 4. Cells were harvested at different time points, counted with trypan blue exclusion and analyzed for apoptosis and cell cycle profile. ***A)*** *Expansion was calculated as the fold induction of trypan blue negative cell yields at days 6 and 0. Data were standardized to control. Mean±SEM expansion is shown (n=9).* ***B)*** *LY or Rapa was added in increasing concentrations. Day 6 yields were standardized to control. Data represent mean±SEM trypan blue negative cell yields of 2 independent experiments with different donors.* ***C)*** *Representative AnnexinV/PI plots from day 5 (n=3).* ***D)*** *Percentages of AnnexinV·PI$^-$ cells were standardized to control. Mean±SEM viability of at least 3 independent experiments with different donors are shown.* ***E)*** *SubG0 cells were excluded from analysis. Representative cell cycle profiles from day 3 (n=3).* ***F)*** *Percentages of viable cells in G0/1 were standardized to control. Mean±SEM cells in G0/1 of up to 3 independent experiments with different donors are shown. * p<0.05, paired Student t-test on original data*

PI3K or mTOR inhibition during terminal differentiation induces specific loss of less differentiated cells

Then, the importance of PI3K and mTOR activity during development of pre-DC into intDC and LC was investigated. Day 6 pre-intDC and pre-LC were isolated by FACSorting and subsequently cultured in the presence or in the absence of LY or Rapa. In both pre-intDC and pre-LC cultures, inhibition of PI3K or mTOR resulted in reduced cell survival by the induction of apoptosis within one day (Figure 3A). Under control conditions, CD1a$^-$CD14$^+$ pre-intDC differentiate to CD1a$^+$CD14$^-$ intDC via an intermediate CD1a$^+$CD14$^+$ state, while CD1a$^+$CD14$^-$ pre-LC remain CD1a$^+$ and acquire Langerin upon terminal differentiation (22). Interestingly, in intDC cultures both PI3K and mTOR inhibition induced a time-dependent relative increase in differentiated cells (Figure 3B,C). Whereas after one day the ratio of differentiated CD1a$^+$ cells and CD1a$^-$CD14$^+$ pre-intDC was similar for all intDC cultures irrespective of PI3K or mTOR inhibition, two and three days incubation with inhibitors resulted in a relative increase in CD1a$^+$ cells compared to control cultures. Also the percentage of Langerin$^+$ cells in end stage LC cultures was increased upon addition of LY or Rapa from day 6 (Figure 3D,E). However, this relative increase was not due to an increased differentiation, as demonstrated by the unchanged CD1a$^+$ and Langerin$^+$ absolute cell numbers (Figure 3F,G). Instead, it resulted from a specific loss of the less differentiated CD1a$^-$ and Langerin$^-$ cells. Thus, PI3K and mTOR activity is required for the survival of pre-intDC and pre-LC, whereas further differentiated cells seem to be unaffected by inhibition of this pathway.

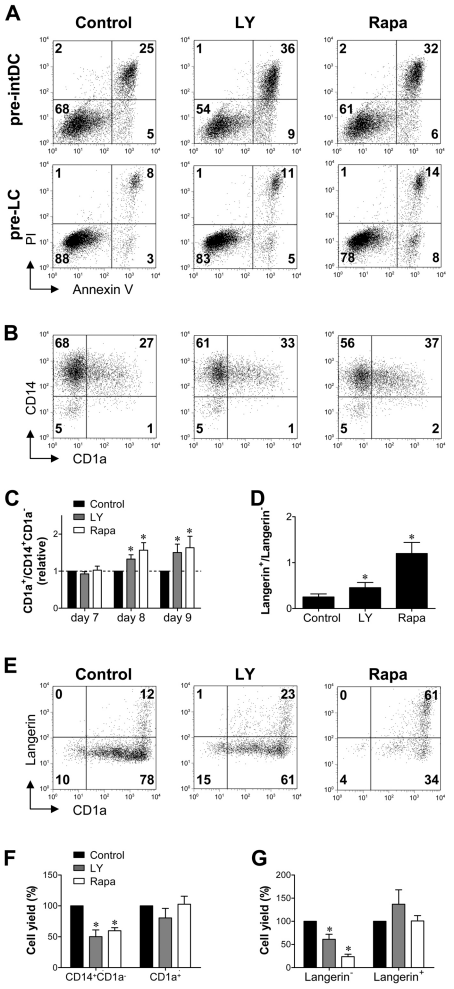

Figure 3. PI3K or mTOR inhibition induces specific loss of less differentiated cells

Pre-intDC and pre-LC were isolated by FACSort at day 6. LY, Rapa or DMSO was added at day 6 and day 10. Cells were harvested at day 7, 8, 9 or 13, counted with trypan blue exclusion and analyzed for apoptosis and CD1a, CD14 or Langerin expression. ***A)*** *Representative AnnexinV/PI plots from intDC (n=9) and LC (n=4) cultures at day 7.* ***B)*** *Representative FACS plots from intDC cultures at day 8 (n=7). Only viable cells, gated on FSC/SSC, are shown.* ***C)*** *Percentages CD1a$^+$ (CD14$^+$CD1a$^+$ and CD14$^-$CD1a$^+$ together) and CD14$^+$CD1a$^-$ cells in intDC cultures were determined and the ratio was calculated. Shown are mean±SEM ratios, standardized to control.* ***D)*** *Percentages Langerin$^+$ and Langerin$^-$ cells in day 13 LC cultures were determined and the ratio was calculated. Mean±SEM ratios are shown (n=7).* ***E)*** *Representative FACS plots from LC cultures at day 13 (n=7). Only viable cells are shown.* ***F)*** *Day 9 yields of CD14$^+$CD1a$^-$ and CD1a$^+$ cells in intDC cultures were calculated and standardized to control. Shown are mean±SEM relative cell yields (n=6).* ***G)*** *Day 13 yields of CD1a$^+$Langerin$^-$ and CD1a$^+$Langerin$^+$ cells in LC cultures were calculated and standardized to control. Shown are mean±SEM relative cell yields (n=7). * p<0.05, Wilcoxon signed rank test on original data*

Terminally differentiated DC are less dependent on PI3K and mTOR activity than pre-DC

To further investigate this apparent reduction in dependency, the role of PI3K and mTOR in the survival of pre-DC and terminally differentiated DC was compared. LY or Rapa were added to pre-intDC and pre-LC, or to intDC and LC generated from these pre-DC. In contrast to pre-intDC (Figure 3A), terminally differentiated intDC survived in the presence of LY or Rapa as shown by an AnnexinV/PI staining comparable to control cultures (Figure 4A,B). Similarly, LY- and Rapa-induced apoptosis was reduced in LC cultures compared to pre-LC cultures (Figure 4C), leading to a similar survival in inhibitor and control cultures (Figure 4A). Thus, whereas pre-intDC and pre-LC highly depend on PI3K and mTOR activity, these proteins seem redundant for the survival of terminally differentiated DC.

To investigate whether these differences in survival regulation could be explained by changes in the expression of known regulators of apoptosis, RT-MLPA was used to compare the mRNA composition of pre-intDC and intDC and pre-LC and LC. Large differences in relative expression profiles were induced during development of both subsets (Table 1). Some of these changes, including the increased relative expression of anti-apoptotic IAP

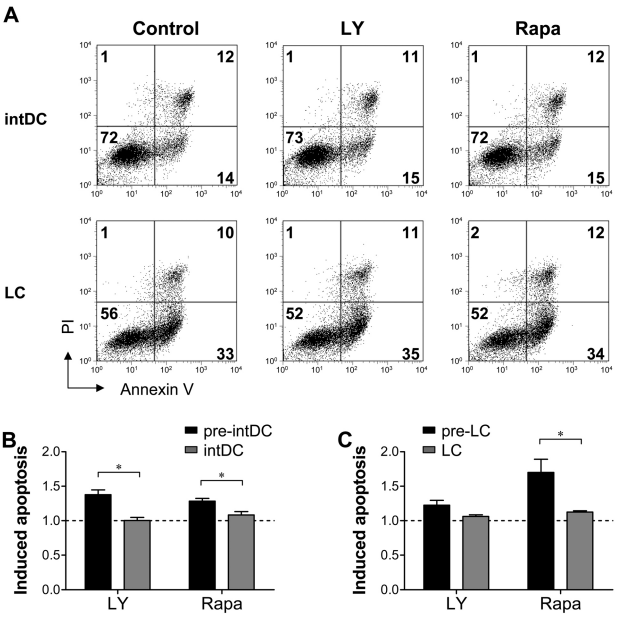

Figure 4. PI3K and mTOR activity are not critical for the survival of terminally differentiated DC

Pre-intDC and pre-LC were isolated by FACSort at day 6 and further cultured towards intDC and LC. LY, Rapa or DMSO was added from day 6 or 13. Cells were harvested and analyzed for apoptosis one day after addition of inhibitors. Induced apoptosis represents the quotient of apoptosis in inhibitor cultures and spontaneous apoptosis. ***A)*** *Representative AnnexinV/PI plots at day 14 (n=5).* ***B)*** *Shown is mean±SEM induced apoptosis in intDC cultures (n=5).* ***C)*** *Shown is mean±SEM induced apoptosis in LC cultures (n=3-5). * p<0.05, paired Student t-test on original quotients*

family members and the reduced expression of pro-apoptotic proteins such as APAF, could reflect an overall reduced susceptibility to apoptosis. However, other differences, such as the reduced relative expression of the mTOR-regulated Mcl-1, suggest a specific reduction in PI3K/mTOR dependency for survival.

PI3K-PKB-mTOR signaling regulates CD34-derived myeloid DC development

Although treatment with LY or Rapa induced overlapping results (Figure 1-4), it remains possible that the observed effects of LY and Rapa treatment are due to unrelated consequences of PI3K and mTOR inhibition. Since PI3K-induced mTOR activation is mediated by the activation of PKB (11), the relation between LY- and Rapa-induced effects on DC development was investigated by transducing $CD34^+$ HPC with a bicistronic retroviral DNA construct co-expressing eGFP and a constitutively active form of PKB (myrPKB). HPC transduced with myrPKB showed increased expansion in relation to progenitors transduced with the control construct (Figure 5A) and the viability of pre-DC expressing myrPKB was increased compared to control cells (Figure 5B). Furthermore, LY-induced apoptosis was completely abolished in myrPKB-transduced cells, while apoptosis induced by mTOR inhibition was unaffected (Figure 5C), placing PKB downstream of PI3K and upstream of mTOR. Moreover, myrPKB was able to rescue cells from apoptosis induced by deprivation of GM-CSF (Figure 5D), a cytokine known to trigger PI3K activation and involved in myeloid cell survival (34-35). Together, these data indicate that the roles of PI3K and mTOR in CD34-derived myeloid DC development can be ascribed to their function in the PI3K-PKB-mTOR signaling module and suggest that GM-CSF stimulation results in activation of this pathway in vitro.

Constitutive PKB activation increases CD34-derived myeloid DC development in vivo

To investigate human CD34-derived DC development in vivo, myrPKB- or control- transduced human $CD34^+$ HPC were transplanted into $\beta2$-microglobulin$^{-/-}$NOD/SCID mice. Six weeks after injection, human myeloid DC originating from transduced HPC could be recognized in the bone marrow by their expression of eGFP and CD1c. The proportions of $eGFP^+CD1c^+CD20^-$

Table 1. Differentiation-induced changes in genes regulating apoptosis

Type	Gene	intDC				LC			
		pre-DC	DC	p	Δ	pre-DC	DC	p	Δ
Bcl-2-like	A1/Bfl-1	1.45	6.12	0.01	**2.08**	1.76	6.96	0.01	**1.99**
anti-apoptotic	Bcl-W	*<0.5*[*]	*<0.5*			*<0.5*	*<0.5*		
	Bcl-XL	3.73	2.08	0.01	-0.85	2.93	0.92	0.04	**-1.67**
	Bcl-2	*<0.5*	*<0.5*			*<0.5*	*<0.5*		
	Mcl1-L	7.10	3.42	0.00	**-1.05**	5.08	3.50	0.00	-0.54
Bax-like	BAK	1.80	0.49[**]	0.00	**-1.87**	1.76	0.33	0.10	-2.40
pro-apoptotic	Bax1	11.69	4.62	0.00	**-1.34**	11.47	8.23	0.10	-0.48
	Bax2	*<0.5*	*<0.5*			*<0.5*	*<0.5*		
	Bcl-GS	*<0.5*	*<0.5*			0.00	0.66	0.09	
	Bcl-Rmb	3.40	1.40	0.00	**-1.28**	3.47	1.02	0.01	**-1.77**
	Mcl1-S	0.94	0.70	0.42	-0.41	0.48	0.68	0.60	0.51
BH3-only	Bad	*<0.5*	*<0.5*			*<0.5*	*<0.5*		
pro-apoptotic	Bid	4.63	5.06	0.54	0.13	5.69	2.97	0.06	-0.94
	Bik	*<0.5*	*<0.5*			*<0.5*	*<0.5*		
	Bim	4.02	5.16	0.23	0.36	3.58	6.58	0.01	0.88
	Bmf	0.82	0.64	0.37	-0.37	0.76	0.66	0.92	-0.20
	NIP3	0.70	0.24	0.01	**-1.56**	*<0.5*	*<0.5*		
	Nix	1.71	1.44	0.19	-0.24	0.92	1.14	0.26	0.30
	Harakiri	*<0.5*	*<0.5*						
	MAP-1	0.59	0.30	0.06	-0.97	*<0.5*	*<0.5*		
	Noxa	1.82	3.91	0.07	1.11	2.33	3.42	0.51	0.55
	Puma	*<0.5*	*<0.5*			0.35	0.52	0.59	0.58
IAP family	Apollon	2.72	1.21	0.00	**-1.16**	2.13	0.94	0.26	-1.19
anti-apoptotic	IAP1	2.15	24.88	0.00	**3.53**	3.70	28.73	0.03	**2.96**
	IAP2	*<0.5*	*<0.5*			*<0.5*	*<0.5*		
	Livin	*<0.5*	*<0.5*			*<0.5*	*<0.5*		
	NIAP	6.39	0.43	0.01	**-3.89**	3.01	0.82	0.35	-1.88
	Survivin	0.75	0.15	0.13	-2.35	3.41	0.15	0.13	-4.48
	XIAP	2.36	0.88	0.03	**-1.42**	2.00	0.53	0.00	**-1.93**
Miscellaneaous	AIF	3.05	0.90	0.00	**-1.76**	3.62	0.64	0.01	**-2.50**
pro-apoptotic	DIABLO	2.69	1.17	0.00	**-1.21**	3.68	1.10	0.06	-1.74
	APAF	9.69	0.93	0.00	**-3.38**	9.32	0.83	0.02	**-3.48**
anti-apoptotic	PI-9	12.36	20.73	0.00	0.75	14.92	15.34	0.97	0.04
	Flip	3.73	4.95	0.45	0.41	3.73	4.48	0.36	0.26

Pre-intDC and pre-LC were isolated by FACSort at day 6. Cells were further cultured and harvested at day 7 or 14, after re-seeding at day 13. Total RNA was isolated and analyzed by RT-MLPA. The expression of 34 apoptosis genes was determined and relative expressions were calculated as percentages of total mRNA. Per gene, the difference of expression (Δ) was calculated as "$\Delta = {}^{2}\log$ (relative expression in DC / relative expression in pre-DC)". $\Delta \geq 1$ and $\Delta \leq -1$, with the difference between pre-DC and DC being statistically significant ($p<0.05$, paired Student t-test), are seen as biologically significant and shown in bold (n=3-4).

** Values below 0.5 are subject to fluctuation. Therefore, when values for both pre-DC and DC were 0.5 or lower, no further analysis has been performed.*

*** When only one value was below 0.5, analysis was performed as for all other genes, but care must be taken when interpreting these data.*

human DC in mice transplanted with progenitors expressing constitutively active PKB were enhanced 20-fold compared to mice that had received a transplant of control-transduced HPC (Figure 5E,F). Thus, in vivo CD34-derived human myeloid DC development is augmented by the ectopic expression of constitutively active PKB, which confirms the role of PI3K-PKB-mTOR signaling in the development of these cells.

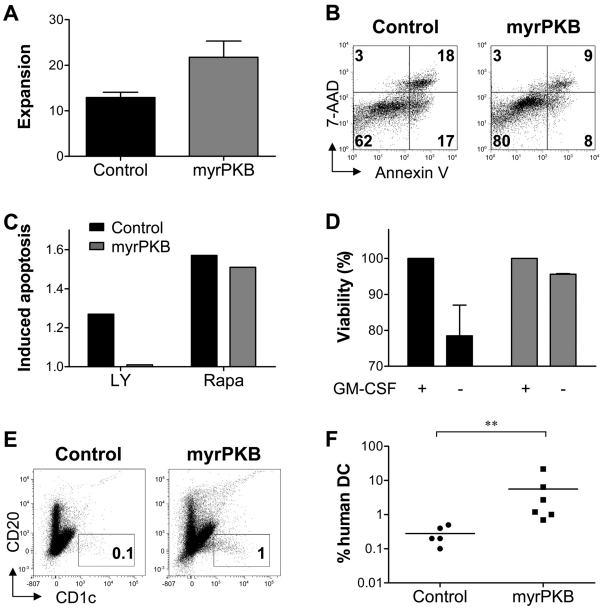

Figure 5. Constitutively active PKB increases CD34-derived myeloid DC development in vitro and in vivo

CD34⁺ HPC were retrovirally transduced with a bicistronic retroviral DNA contruct co-expressing eGFP and myrPKB or with a control construct expressing only eGFP. ***A)*** *Cells were counted with trypan blue exclusion and analyzed for the percentage of eGFP⁺ cells 2 and 5 days after transduction. Numbers of eGFP⁺ cells were calculated and the fold induction was determined. Shown are mean±SEM fold inductions (n=3).* ***B)*** *eGFP⁺ cells were isolated by FACSorting at day 7 and further cultured under control conditions. At day 11, cells were harvested and viability was determined. Representative AnnexinV/7-AAD plots are shown (n=4).* ***C)*** *LY, Rapa or DMSO was added to day 7 FACSorted eGFP⁺ cells. At day 8, cells were harvested and analyzed for apoptosis. Induced apoptosis was calculated as for figure 4. A representative experiment is shown (n=2).* ***D)*** *Day 7 FACSorted eGFP⁺ cells were cultured with or without GM-CSF. At day 8, cells were harvested and analyzed for apoptosis. Shown are mean±SEM percentage AnnexinV⁻7-AAD⁻ cells (n=2).* ***EF)*** *Day 3 unsorted cells were intravenously injected into β2-microglobulin⁻/⁻NOD/SCID mice (eGFP: n=5; myrPKB: n=6). Six weeks post-transplantation, the bone marrow was analyzed for the presence of CD1c⁺CD20⁻ human myeloid DC within the eGFP⁺ fraction. Representative FACS plots gated on eGFP⁺ cells (E) and percentage CD1c⁺CD20⁻ myeloid DC of eGFP⁺ cells per mouse (F) are shown. ** p<0.01, Mann-Whitney U test*

A role for PI3K and mTOR in functional, but not phenotypic maturation

While we could show that PI3K and mTOR activity was not critical for the phenotype or survival of terminally differentiated DC, inhibition of PI3K or mTOR activity during either DC differentiation or DC activation has been suggested to negatively affect DC function (13, 36-38). We therefore investigated the functionality of CD34-derived myeloid DC differentiated or activated in the presence of LY or Rapa. Both lectin-mediated and fluid phase endocytosis, as investigated by incubation with Dextran^{FITC} and Lucifer Yellow respectively, were reduced in cells generated in the presence of Rapa, while cells generated with LY showed only a slightly reduced Ag uptake capacity (Figure 6). Ag uptake capacity of control CD34-derived myeloid DC was however not affected by the addition of LY or Rapa during incubation with Dextran^{FITC} or Lucifer Yellow (data not shown).

Also the expression of costimulatory molecules was unaffected by PI3K or mTOR inhibition and neither LY nor Rapa affected LPS-induced upregulation of CD83, CD86 or HLA-DR (Figure 7A). However, LY and Rapa did inhibit CCR7 expression (Figure 7A), indicating that this signaling module may be involved in the regulation of lymph node migration. Although only low cytokine concentrations could be measured due to low cell densities, LPS stimulation did induce the production of detectable levels of IL-6, IL-8 and IP-10, which were

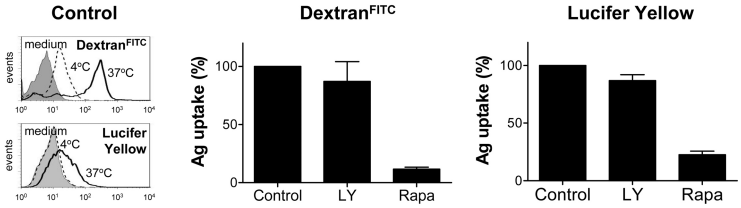

Figure 6. Impaired Ag uptake by cells differentiated in the presence of mTOR inhibition
LY, Rapa or DMSO was added to CD34-derived myeloid DC differentiation cultures at days 0, 4 and 6. Cells were harvested at day 10, thoroughly washed and incubated with Dextran^{FITC} or Lucifer Yellow. Lectin-mediated (Dextran^{FITC}) and fluid phase (Lucifer Yellow) endocytosis were determined after 2 hours of incubation at 37°C. Surface binding was determined by incubation at 4°C. Ag uptake was calculated as "MFI 37°C – MFI 4°C". Data shown are mean±SEM Ag uptake standardized to control (n=2).

reduced by the addition of LY and Rapa during either differentiation or activation of the cells (Figure 7B,C). Also the secretion of IL-10 and TNF-α depended on intact PI3K and mTOR activity (Figure 7C). Secretion of IL-12p70, which could only be detected after stimulation with CD40L, was also reduced by the presence of LY or Rapa during either differentiation or activation (Figure 7C and data not shown). In accordance with the stronger effects of Rapa compared to LY (Figure 7), T cell proliferation induced by myeloid DC pre-treated with Rapa,

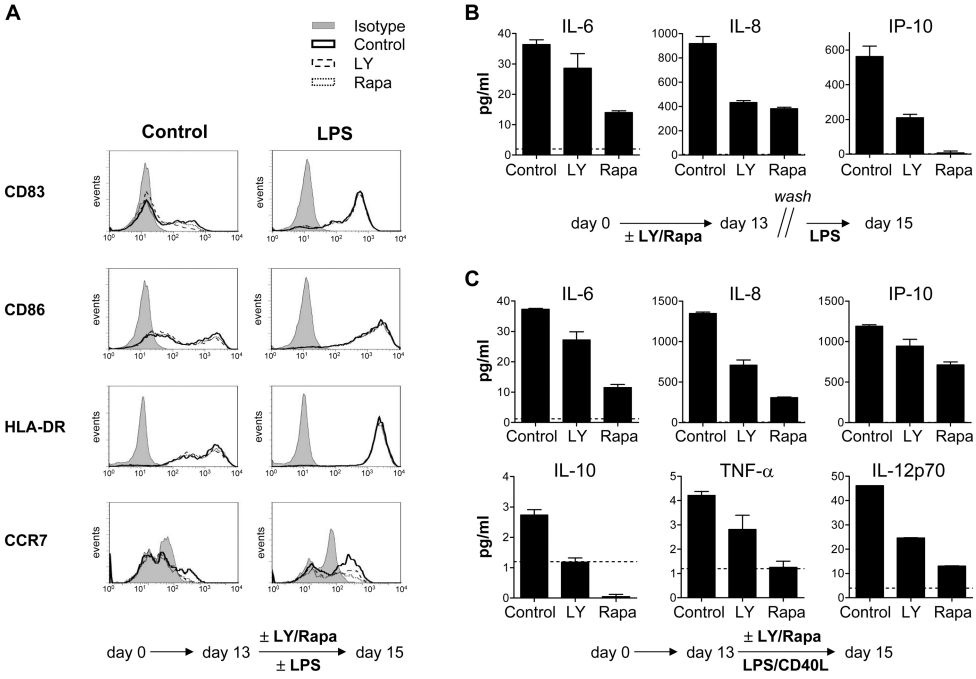

Figure 7. PI3K signaling is required for functional maturation
CD34-derived myeloid DC were differentiated under control conditions and subsequently stimulated with LPS or CD40L-transfected L-cells in the presence or absence of LY or Rapa (2 hours pre-incubation) (A,C) or differentiated in the presence or absence of LY or Rapa, thoroughly washed and stimulated with LPS without inhibitors (B). ***A)*** *LPS- or non-stimulated cells were analyzed for the expression of CD83, CD86, HLA-DR (cells harvested at day 14; n=4) and CCR7 (n=2). Viable cells were gated on FSC/SSC. Representative FACS plots are shown.* ***B)*** *LPS-induced IL-6, IL-8 and IP-10 production was determined by ELISA (n=2). Shown are mean±SD of duplicate cultures of a representative experiment. Dotted line shows detection limit of the assay.* ***C)*** *IL-6, IL-8, IL-10, IP-10 and TNF-α concentrations were determined in supernatants from LPS cultures. IL-12p70 concentration was determined in supernatants from CD40L-stimulated cultures. Cytokine concentrations were determined by luminex (IL-6, IL-10, TNF-α; supernatants harvested at day 14; n=3) or ELISA (IL-8, IP-10, IL-12p70; n=2). Shown are mean±SD of duplicate cultures of a representative experiment. Dotted line shows detection limit of the assay.*

but not LY, was reduced compared to control DC (Figure 8). Only when using DC treated with Rapa during activation, this reduced proliferation was clearly associated with reduced IFN-γ concentrations (Figure 8). A reduction in IL-5 production was also observed in co-cultures of T cells with Rapa-treated DC, while IL-10 concentrations were similar in all cultures (Figure 8). Like for the non-stimulated DC, neither LY nor Rapa affected the survival of LPS-activated DC (data not shown), indicating that the effects on DC function were not caused by reduced DC viability. These data show that while PI3K and mTOR activity are dispensable for the phenotype and survival of terminally differentiated CD34-derived myeloid DC, these proteins play an important role in the functionality of these cells.

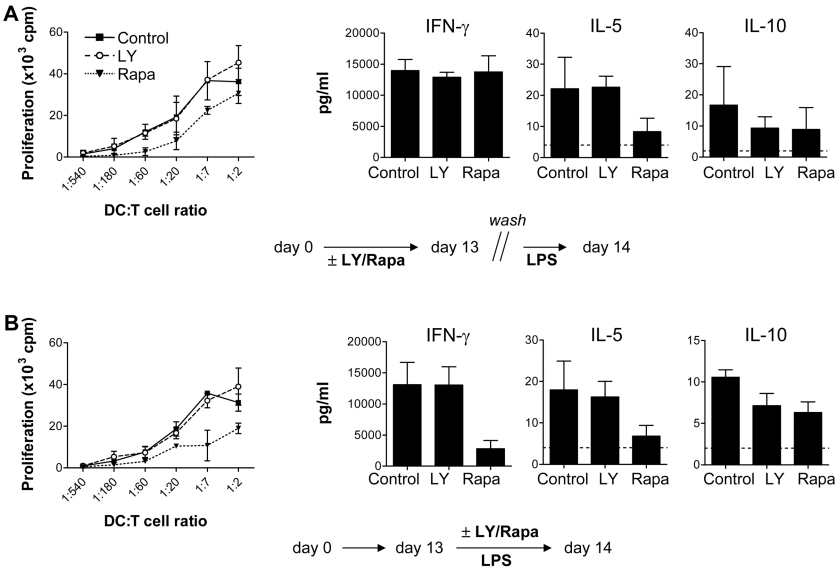

Figure 8. PI3K signaling is required for myeloid DC function

CD34-derived myeloid DC were differentiated in the presence or absence of LY or Rapa, thoroughly washed and stimulated with LPS without inhibitors (A) or differentiated under control conditions and subsequently stimulated with LPS in the presence or absence of LY or Rapa (2 hours pre-incubation) (B). After 18 hours LPS stimulation, cells were thoroughly washed and used in an allogeneic MLR (n=2). T cell proliferation was quantified by incubating the cells with [methyl-^{3}H]thymidine during the last 18 hours of 6-day cultures. ELISA determined IFN-γ, IL-5 and IL-10 concentrations in supernatants harvested at day 5 (DC:T cell ratio 1:2). Data represent mean±SD of triplicate cultures of a representative experiment.

Discussion

DC play a crucial role in the induction and regulation of immunity. Despite the requirement of DC development and survival for both immunogenic and tolerogenic responses, the molecular mechanisms regulating these processes are still largely undefined. In the present study, we showed that PI3K-PKB-mTOR signaling is required for proliferation and survival, but not differentiation, of human CD34-derived myeloid DC. Inhibition of PI3K-PKB-mTOR signaling reduced the in vitro development of CD34-derived myeloid DC, while activation of this pathway increased DC development both in vitro and in vivo. Although DC phenotype was not affected by inhibition of this signaling module, its activity was required for the generation of fully functional DC.

We and others have described reduced DC yields following Rapa-treatment during murine and human myeloid DC development (19, 38-39). In contrast to mo-DC development, the

development of myeloid DC from human CD34+ progenitors involves not only differentiation and survival, but also proliferation. The reduced intDC and LC yields found upon inhibition of PI3K or mTOR activity were partly caused by a block in cell cycle progression during the development of pre-DC. An increase of cells in G0/1 has also been reported for other cell types in which inhibition of PI3K-PKB-mTOR signaling caused a G1 cell cycle arrest (8, 40-42). The LY- or Rapa-induced apoptosis could be either a direct consequence of cell cycle arrest or an independent process. A combined reduction in cell cycling and survival has also been reported in developing erythrocytes (8), but is not a pre-requisite in all cell types (19, 41). Since only minimal proliferation was present from the stage of pre-DC while these cells did undergo apoptosis upon PI3K or mTOR inhibition, it appears that the LY- and Rapa-induced effects on proliferation and survival are independent consequences of reduced signaling along this pathway.

PI3K signaling plays a critical role in the regulation of survival in various hematopoietic cell types, including murine bone marrow-derived DC and human mo-DC (8, 18, 43). Also during CD34-derived myeloid DC development, survival was decreased upon inhibition of PI3K or mTOR activity and increased in pre-DC with increased activity of this signaling module by transduction with myrPKB. Whereas CD34-derived myeloid pre-DC survival required PI3K-PKB-mTOR signaling, terminally differentiated CD34-derived DC survived without active PI3K or mTOR. This lack of apoptosis induction by PI3K or mTOR inhibition in CD34-derived intDC is in sharp contrast to mo-DC, which share phenotypic and functional characteristics with CD34-derived intDC, but die upon inhibition of this pathway (19, 44). Moreover, whereas CD34-derived pre-DC require mTOR for their survival, monocytes, the direct precursors of mo-DC, are insensitive to Rapa (19, 44) (Figure 9). These disparities in survival signaling challenge the hypothesis that DC developing from monocytes and from direct DC precursors are completely identical (45).

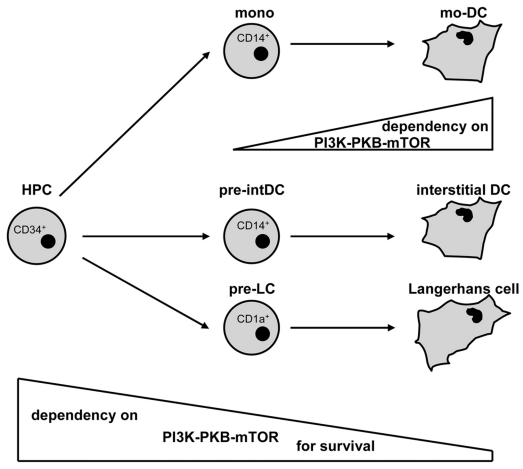

Figure 9. The role of PI3K-PKB-mTOR signaling in human myeloid DC subset development.
CD34+ HPC differentiate into intDC and LC via subset specific pre-DC. Early in CD34-derived myeloid DC development, PI3K-PKB-mTOR signaling is required for proliferation and survival. The dependency on this signaling module for survival is decreasing upon differentiation, with terminally differentiated intDC and LC survival being unaffected by inhibition of PI3K or mTOR. In contrast, mTOR activity has been reported to be crucial for survival of mo-DC, while their direct precursors, monocytes, are insensitive to mTOR inhibition (19, 44).

Although the effects of PI3K and mTOR inhibition on survival and proliferation could theoretically be unrelated, this seems unlikely. The lack of effects induced by only mTOR or only PI3K inhibition as well as the complementary consequences of PI3K/mTOR inhibition and increased PKB activity suggest that the observed effects reflect a critical role of the PI3K-PKB-mTOR signaling module. Also the myrPKB-induced rescue from GM-CSF withdrawal- or PI3K inhibition-, but not mTOR inhibition-induced apoptosis supports the suggestion that PI3K regulates CD34-derived myeloid DC development via the activation

of PKB and mTOR.
The apparently absent PI3K-PKB-mTOR dependency of terminally differentiated DC could reflect a generally reduced apoptosis sensitivity of these cells compared to their precursors, for example due to the decreased APAF expression in these cells (46). Alternatively, other signaling pathways than those including mTOR might be involved in the survival regulation of terminally differentiated myeloid DC. This hypothesis is supported by the observation that the expression of the anti-apoptotic Bcl-2-like proteins Mcl-1 and Bcl-XL is reduced in differentiated CD34-derived DC compared to their precursors (Table 1). Although the regulation of these proteins by mTOR is at the translational level (47-48), the reduced mRNA expression suggests a diminished role in survival regulation and thus a reduced requirement of mTOR-mediated translational control of these proteins. The increase in Bfl-1/A1 expression, a Bcl-2 family member with similar functions as Mcl-1 but regulated by NF-κB (49-50), may point towards a role for other signaling pathways in the survival regulation of differentiated CD34-derived myeloid DC.
Since the surviving cells demonstrated normal pre-DC and DC phenotype, PI3K-PKB-mTOR signaling appears to be redundant for differentiation of CD34-derived myeloid DC. However, tolerogenic effects of Rapa addition during DC differentiation have been reported (38, 51) and in particular activation-induced cytokine secretion has been shown to be regulated by PI3K and mTOR (13-16, 36, 52). In accordance, the function of CD34-derived myeloid DC differentiated or activated in the presence of PI3K or mTOR inhibition was inhibited. As previously reported for mouse bone-marrow derived DC and human mo-DC (39, 53), human CD34-derived myeloid DC differentiated in the presence of Rapa showed reduced macropinocytosis and lectin-mediated endocytosis. In contrast, LY or Rapa did not affect Ag uptake by control DC when inhibitor and Ag were added simultaneously (data not shown), indicating that neither PI3K nor mTOR was directly involved in the regulation of endocytosis. Rather, mTOR inhibition during differentiation seems to induce development of altered DC with relatively poor Ag uptake capacity. Also the reduced cytokine secretion by DC generated in the presence of LY or Rapa indicates the development of alternatively functioning DC. However, since LY and Rapa also inhibited DC function when added during activation, we cannot fully exclude the possibility that these effects result from ongoing PI3K- and/or mTOR inhibition during activation. Although both LY and Rapa inhibited LPS-induced DC function, LY did so to a lesser extent, which could explain the reduced T cell stimulatory capacity observed for Rapa- but not LY-treated DC. These findings seem contradictory to some studies describing repression of cytokine production by PI3K and mTOR in mo-DC (16, 36), but are in accordance with others (38, 54-56). Together, these data indicate that although PI3K-PKB-mTOR signaling is not required for the acquisition of a DC phenotype or the survival of terminally differentiated CD34-derived myeloid DC, this pathway may still be involved in the regulation of other important processes in these cells.
The molecular mechanism underlying the impaired response to activation signals following mTOR inhibition is relatively unknown. Minor changes in TLR4 mRNA expression were observed (data not shown), which might provide an explanation for the reduced response to LPS. However, as LPS induced normal upregulation of costimulatory molecule expression, loss of TLR4 stimulation cannot fully account for the diminished function observed after mTOR inhibition. While the role of mTOR in the regulation of proliferation and survival is mainly ascribed to translational control (11, 57), cytokine production has been suggested to be regulated on the transcriptional level (15, 58-59). However, the exact mechanisms of the processes involved remain to be elucidated.
While the decreased T cell proliferation induction by DC treated with Rapa could result from the induction of regulatory T cell differentiation as suggested before (38, 51), FOXP3 expression by these T cells was unchanged compared to control (data not shown). Whether

the altered secretion of Th1 and Th2 cytokines after co-culture with Rapa-treated DC only reflects reduced T cell numbers or results from changed Th1 and Th2 differentiation as shown for murine Rapa-differentiated DC (37) remains to be determined. Furhermore, although the reduced IL-5 secretion but unaffected IFN-γ concentrations found in T cell cultures stimulated with Rapa-differentiated DC might suggest skewing of Th differentiation, this should be investigated further. In addition to reduced DC-T cell interaction as a consequence of mTOR inhibition in DC, the reduced CCR7 expression following LY- or Rapa-treatment may also have consequences for the induction of immune responses. These data suggest that lymph node migration requires intact activity of the PI3K-mTOR signaling module, as has been suggested before (60).
In conclusion, PI3K-PKB-mTOR signaling is required for human CD34-derived myeloid DC development by regulating the proliferation and survival of DC precursors. While this signaling module is redundant for differentiation and phenotypic maturation as well as survival of terminally differentiated CD34-derived intDC and LC, its activity is required for the generation of fully functional DC. These findings could be used as a strategy to manipulate DC subset distribution and function to regulate immunity.

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AN ASSOCIATION BETWEEN APAF-1 LEVELS AND GM-CSF DEPENDENCY AT DISTINCT MYELOID DENDRITIC CELL DIFFERENTIATION STADIA

3
APPENDIX

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Rationale

Survival of dendritic cells (DC) and their precursors is of crucial importance for the development of immune responses. Specific expression patterns of known apoptosis regulators such as Bcl-2, Bcl-XL, Bax and Bak have been associated with variations in lifespan between distinct DC subsets (1). Differential regulation of cell survival has also been described for DC precursors and DC (2-4). For human CD34-derived interstitial DC (intDC) and Langerhans cells (LC), we have recently shown that whereas precursor DC (pre-DC) require activity of the PI3K-PKB-mTOR pathway to survive, this pathway is redundant for the survival of terminally differentiated intDC and LC (2). It is possible that the uptake of the pharmacological inhibitors used to modulate the activity of this pathway is altered in DC. However, this finding could also reflect either a shift in signaling pathways regulating survival or a generally reduced sensitivity to apoptosis of DC compared to pre-DC. Here, we aimed to further elucidate the observed differences in CD34-derived pre-DC and DC survival regulation.

Materials and Methods

Generation of CD34-derived interstitial DC and Langerhans cells
Umbilical cord blood samples were obtained ex-uterine according to legal guidelines. $CD34^+$ hematopoietic progenitor cells were isolated and differentiated towards intDC and LC as described previously (2). Briefly, $CD34^+$ cells, isolated through positive selection using anti-CD34-coated microbeads (Miltenyi Biotec GmBH, Bergish Gladbach, Germany), were cultured in complete medium containing RPMI-1640 (Invitrogen, Breda, The Netherlands), 8% heat inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 10 mM Hepes (Invitrogen), 2 mM L-glutamine (Lonza, Breda, The Netherlands), 50 µM β-mercaptoethanol (Merck, Darmstadt, Germany) and penicillin/streptomycin (Invitrogen), supplemented with 100 ng/ml GM-CSF (Leukine sargramostim, Bayer HealthCare, Seattle, WA), 25 ng/ml stem cell factor (SCF, PeproTech, London, UK), 2.5 ng/ml TNF-α (R&D systems, Abingdon, UK) and 5% heat inactivated AB^+ pooled human serum (Lonza). At day 6, cultures were harvested, incubated with fluorochrome-conjugated antibodies against CD1a (HI149, BD Biosciences, Breda, The Netherlands) and CD14 (MΦP9, BD Biosciences), life gated and sorted into $CD14^+CD1a^-$ pre-intDC and $CD14^-CD1a^+$ pre-LC fractions using a FACSAria (BD Biosciences). Pre-intDC and pre-LC were then cultured separately in complete medium supplemented with 100 ng/ml GM-CSF.

Apoptosis detection
Apoptosis was detected by determination of phosphatidyl serine exposure. Cells were harvested, washed in Annexin buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), and incubated with Annexin V-FITC (BD Biosciences) for 30 minutes on ice. Assessment was performed using a FACSCalibur or FACSCanto II (BD Biosciences) and data were analyzed using FlowJo software (www.flowjo.com).

Western blot
Total cell lysates were produced using laemmli sample buffer (0.12 M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 µg/µl bromophenol blue and 35 mM β-mercaptoethanol) and boiled for 5 minutes. Equal amounts of total protein, determined by Lowry method, were separated by 8% SDS-PAGE, transferred to an Immobilon-FL transfer membrane (Millipore, Bedford, MA) and incubated with Odyssey blocking buffer (Westburg, Leusden, The Netherlands) for 30 minutes before probing with antibodies against human APAF-1 (rabbit polyclonal, Cell Signaling Technology, Danvers, MA), caspase-9 (rabbit polyclonal, Cell Signaling Technology) or β-tubulin (mouse monoclonal, Sigma-Aldrich, St. Louis, MO). Subsequently, blots were incubated with goat-anti-mouse IgG IRDye 680 (Westburg) or goat-anti-rabbit IgG IRDye 800CW (Westburg). Blots were scanned using an Odyssey Imager (Li-cor, Lincoln, NE) and analyzed and quantified by Odyssey software (Li-cor).

Results and Discussion

We have previously reported significant differences in mRNA expression profiles of apoptosis regulators between pre-DC and DC (2). While some of these changes suggest a specific reduction in PI3K/mTOR dependency for survival, other differences might reflect an overall reduced susceptibility to apoptosis of DC compared to pre-DC. To investigate whether the reduced requirement for GM-CSF-induced PI3K-PKB-mTOR signaling in DC could be due to a general reduced GM-CSF-dependency, day 6 pre-intDC and pre-LC and day 13 intDC and LC were cultured in the presence or absence of GM-CSF. Whereas GM-CSF deprivation induced apoptosis of pre-DC within 18 hours, as demonstrated by the increased Annexin V binding, the viability of terminally differentiated DC was similar in cultures with or without GM-CSF (Figure 1). The reduced need for pro-survival signaling in terminally differentiated DC appears relevant in vivo, as DC reside in tissues that may lack efficient production of survival factors. Although it appears unlikely that circulating pre-DC specifically require GM-CSF since the serum concentration is normally low (5), survival of these cells is probably sustained by additional pro-survival factors. Nevertheless, these data clearly indicate a general increased need for pro-survival signaling in pre-DC compared to DC.

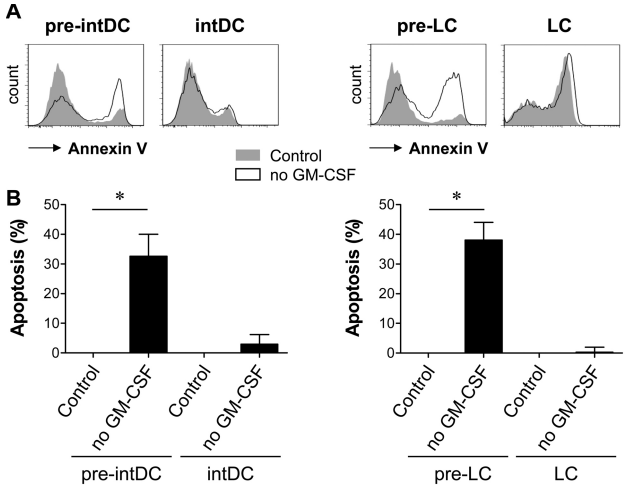

Figure 1. Pre-DC, but not DC, require GM-CSF to survive

*Day 6 pre-DC and day 13 DC generated from the same donor were harvested, thoroughly washed and subsequently cultured in the presence or absence of GM-CSF. Annexin V staining was analyzed after 18 hours. Apoptosis was determined as the percentage of cells staining for Annexin V, and standardized to control by subtracting the percentage of apoptotic cells in control cultures from the percentage of apoptotic cells in cultures without GM-CSF. FACS plots (A) and mean±SEM apoptosis standardized to control (B) are representative for 6 (intDC) and 5 (LC) independent experiments with different donors. * p<0.05, paired Student t-test*

Comparing the mRNA composition of pre-DC and DC (2) resulted in the identification of candidate genes whose differential expression in pre-DC and DC could explain increased apoptosis resistance in DC. DC were found to express high mRNA levels of inhibitor of apoptosis 1 (IAP1). However, the abundant expression of this member of the IAP family, which suppresses apoptotic death by intervening with the caspase pathways at different levels (6), could not be confirmed at the protein level (data not shown).

Another potential candidate was apoptotic protease-activating factor-1 (APAF-1), a pro-apoptotic protein with a central role in stimulating the mitochondrial pathway to caspase activation (7). Cellular stress, damage, growth factor deprivation or other cell intrinsic stimuli result in release of cytochrome c from mitochondria. In the cytosol, cytochrome c induces oligomerization of APAF-1, leading to the recruitment and subsequent activation of caspase-9 in a complex called the apoptosome. This initiates the activation of effector caspases, resulting in cleavage of cellular proteins and eventually causing demolition of the cell (8). Supporting the mRNA expression data, the expression of APAF-1 protein was high in pre-DC, but strongly diminished in DC (Figure 2). In contrast, caspase-9 expression did

not change (Figure 2A). Although these data suggest a reduced ability for the formation of the apoptosome by terminally differentiated DC compared to pre-DC, the formation of this complex could be affected by other factors, such as the levels of cytochrome c released. Further analysis of the efficiency of apoptosome formation in pre-DC compared to DC is therefore required.

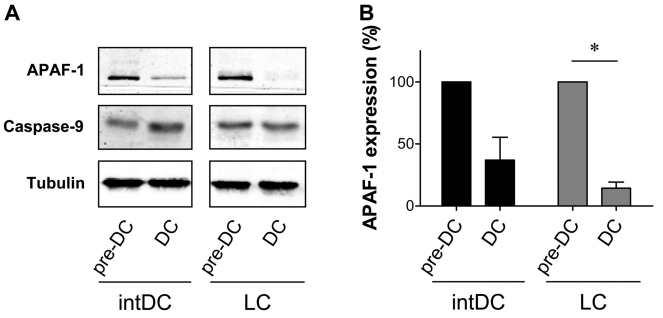

Figure 2. Decreased APAF-1 expression in DC compared to pre-DC
*Expression of APAF-1, caspase-9 and tubulin was determined in total cell lysates of pre-intDC, intDC, pre-LC and LC. APAF-1 expression was normalized to tubulin and standardized to pre-DC. A representative experiment (A) and mean±SEM APAF-1 expression (B) are shown (n=3). * p<0.05, paired Student t-test*

Specific loss of APAF-1 could provide an explanation for the resistance to apoptosis observed in terminally differentiated DC. Deficient APAF-1 activity is believed to be responsible for the increased survival of leukemic cell lines (9-10), whereas high APAF-1 expression in human primary neutrophils has been suggested to account for efficient apoptosome assembly in these cells (11). Although the importance of APAF-1 in the regulation of DC apoptosis is unclear, mitochondria-dependent apoptosis is believed to be of importance in the regulation of DC survival (12). Furthermore, cytochrome c injection induced death of DC of control but not APAF-1-deficient mice, indicating that APAF-1-dependent apoptosis plays a role in DC (13). It is therefore tempting to speculate that the low APAF-1 expression levels in terminally differentiated DC are responsible for the reduced need for GM-CSF-mediated survival signaling. However, a direct link between these observations remains to be established.
In conclusion, CD34-derived intDC and LC are less dependent on GM-CSF and show a reduced expression of APAF-1 compared to pre-DC. These data suggest that altered apoptosis sensitivity is at least partially responsible for differences in CD34-derived pre-DC and DC survival regulation.

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4

A NONREDUNDANT ROLE FOR CANONICAL NF-κB IN HUMAN MYELOID DENDRITIC CELL DEVELOPMENT AND FUNCTION

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Abstract

The plastic role of dendritic cells (DC) in the regulation of immune responses has made them interesting targets for immunotherapy, but also for pathogens or tumors to evade immunity. Functional alterations of DC are often ascribed to manipulation of canonical NF-κB activity. However, although this pathway has been linked to murine myeloid DC biology, a detailed analysis of its importance in human myeloid DC differentiation, survival, maturation and function is lacking. The myeloid DC subsets include interstitial DC (intDC) and Langerhans cells (LC). Here, we investigated the role of canonical NF-κB in human myeloid DC generated from monocytes (mo-DC) or $CD34^+$ progenitors (CD34-mDC). Inhibition of NF-κB activation during and after mo-DC, CD34-intDC or CD34-LC differentiation resulted in apoptosis induction associated with caspase-3 activation and loss of mitochondrial transmembrane potential. Besides regulating survival, canonical NF-κB activity was required for the acquisition of a DC phenotype. Despite phenotypic differences however, Ag uptake, costimulatory molecule and CCR7 expression as well as T cell stimulatory capacity of cells generated under NF-κB inhibition were comparable to control DC, indicating that canonical NF-κB activity during differentiation is redundant for the development of functional APC. However, both mo-DC and CD34-mDC functionality was reduced by NF-κB inhibition during activation. In conclusion, canonical NF-κB activity is essential for the development and function of mo-DC as well as CD34-mDC. Insight in the role of this pathway may help in understanding how pathogens and tumors escape immunity and aid in developing novel treatment strategies aiming to interfere with human immune responses.

Introduction

Dendritic cells (DC) are professional antigen (Ag) presenting cells that play a crucial role in the induction of immunity as well as tolerance (1). The DC subtype, its maturation state and the lifespan of the Ag-bearing DC together determine the type of the initiated immune response (2-5). Based on surface markers, localization, functional abilities and ontogeny, a large variety of DC subsets can be recognized (6).

The myeloid DC subtypes are known for their superior T cell priming ability compared to for example plasmacytoid DC (7). Their ability to induce both immunogenic and tolerogenic immune responses has made them interesting tools for treatment of infections, cancer, autoimmune diseases and allograft rejection (8). Much research has focused on the active manipulation of DC, either to augment favorable immunity or to suppress unwanted immune activation (9-14). In addition, pathogens and tumors often escape immunity via modulation of DC immunogenicity (15-19). In many cases, the functional alterations of DC by drugs, pathogens or tumor-derived factors are ascribed to manipulation of NF-κB activity.

NF-κB/Rel transcription factor family members are expressed at relatively high levels in DC (20). In mammals, they exists as homo- or heterodimers of five distinct proteins including RelA (p65), c-Rel, RelB, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) (21). All these proteins have been knocked out in mice and deficiency of RelB or a combined deficiency of RelA and p50 or c-Rel and p50 all result in DC loss (22-23). The noncanonical transcription factor RelB has further been shown to have a critical role in DC maturation and immunogenicity (22, 24-27). Canonical NF-κB activation, which involves phosphorylation and subsequent degradation of the inhibitory IκB proteins (IκBs) enabling nuclear translocation of RelA, c-Rel and/or p50 dimers, has also been associated with activation-induced DC maturation and function (28-31).

Although knockout studies in mice have elegantly shown a loss of functional DC in the absence of canonical NF-κB proteins, these experiments cannot be directly translated to the human situation. Furthermore, they do not allow separate analysis of the processes involved in DC biology. The exact mechanisms affected by the loss of NF-κB activity therefore remain undefined. Effects on survival, differentiation, maturation and function can be investigated independently in vitro. In vivo, DC develop from $CD34^+$ hematopoietic progenitor cells (HPC) via subset specific precursors that are present either as direct DC precursors, pre-DC, or as immune effector cells with their own function in immunity, such as monocytes (6, 32). To study human DC biology in vitro, DC differentiation from monocytes is the most widely utilized model (33). To investigate the development of myeloid DC subsets from a less committed progenitor, $CD34^+$ HPC can be used. Two functionally different myeloid subsets, intDC and LC, develop from $CD34^+$ HPC via their respective pre-DC in independent pathways (34-35), enabling detailed analysis of this DC developmental route.

Studies investigating canonical NF-κB activation in DC development have mainly focused on mouse DC and human monocyte-derived DC (mo-DC). In the present study we investigated the importance of canonical NF-κB activity in three different human myeloid DC subtypes, mo-DC, CD34-derived intDC and CD34-derived LC. A detailed analysis evaluating the role of canonical NF-κB in many different aspects of myeloid DC biology was performed. These data not only confirm previous studies suggesting that canonical NF-κB is involved in mo-DC function, but also show the importance of its activation for the functionality of other human myeloid DC subtypes. Moreover, we provide important information on the crucial role of canonical NF-κB in human myeloid DC differentiation and survival.

Materials and Methods

Reagents
Where indicated the specific canonical NF-κB inhibitors Caffeic Acid Phenethyl Ester (CAPE; 10 μg/ml unless indicated differently; Sigma-Aldrich, St. Louis, MO), BAY 11-7082 (BAY; 1 μM unless indicated differently; Calbiochem, Merck KGaA, Darmstadt, Germany) or AS602868 (AS; 1 μM unless indicated differently; a kind gift from Dr I. Adcock, Imperial College London, UK) were added to the cultures.

Generation of monocyte-derived dendritic cells (mo-DC)
Human monocytes were isolated from buffy coats obtained from healthy donors using Ficoll (GE Healthcare, Uppsala, Sweden) density gradient centrifugation followed by positive selection using anti-CD14-coated microbeads as indicated by the manufacturer (Miltenyi Biotech GmBH, Bergish Gladbach, Germany). As described before (36), DC were generated in 6 days in 6- or 12-well culture plates (Costar, Cambridge, MA) in RPMI-1640 (Invitrogen, Breda, The Netherlands) containing 8% heat inactivated fetal bovine serum (FBS; Sigma-Aldrich) and penicillin/streptomycin (Invitrogen) supplemented with 10 ng/ml GM-CSF (Bayer HealthCare Pharmaceuticals LLC, Seattle, WA) and 10 ng/ml IL-4 (eBioscience, San Diego, CA).

Generation of CD34-derived myeloid dendritic cells (CD34-mDC)
Umbilical cord blood samples were obtained ex-uterine according to legal guidelines. $CD34^+$ hematopoietic progenitor cells were isolated and cultured as described previously (35, 37). In brief, $CD34^+$ cells were isolated from mononuclear fractions through positive selection using anti-CD34-coated microbeads (Miltenyi Biotec GmBH) to a purity of 85-98%. After cryopreservation to standardize differentiation, cells were cultured in complete medium containing RPMI-1640 supplemented with 8% heat inactivated FBS, 10 mM Hepes (Invitrogen), 2 mM L-glutamine (Invitrogen), 50 μM β-mercaptoethanol (Merck, Darmstadt, Germany) and penicillin/streptomycin. From day 0-6, the cells were cultured in complete medium supplemented with 100 ng/ml GM-CSF, 25 ng/ml SCF (PeproTech, London, UK), 2.5 ng/ml TNF-α (R&D systems, Abingdon, UK) and 5% heat inactivated AB^+ pooled human serum (4 different donors, Sanquin, Rotterdam, The Netherlands). Then, cells were harvested, washed and cultured further in complete medium containing only GM-CSF (100 ng/ml). For separate analysis of intDC and LC, CD34-derived subset specific precursors (pre-DC) were sorted using a FACSAria (BD Biosciences). Control cultures were harvested at day 6, incubated with fluorochrome-conjugated antibodies against CD1a (HI149, BD Biosciences, Breda, The Netherlands) and CD14 (MΦP9, BD Biosciences), life gated and sorted into $CD14^+CD1a^-$ (pre-intDC) and $CD14^-CD1a^+$ (pre-LC) fractions.

Ag uptake
Ag uptake was analyzed as described before (38-39). In short, receptor-mediated endocytosis was measured as the cellular uptake of 100 μg/ml dextran-FITC ($Dextran^{FITC}$, 40,000 MW, Molecular Probes, Invitrogen). Approximately 5 x 10^4 DC were incubated for 2 hours at 37°C in the presence of culture medium containing $Dextran^{FITC}$. Negative controls were incubated with $Dextran^{FITC}$ at 4°C. Staining was evaluated by flow cytometry and Ag uptake was calculated by subtracting staining at 4°C from staining at 37°C.

Activation of DC
Terminally differentiated DC were activated by addition of LPS (100 ng/ml; Invivogen, San Diego, CA) to the cultures. Alternatively, cells were co-cultured with CD40L-transfected L cells (L-CD40L) (40) in a DC:L cell ratio of 4:1. Non-transfected L cells (L-Orient) served as control cells. During activation, cells were incubated in the presence of 10 ng/ml GM-CSF and 10 ng/ml IL-4 (mo-DC) or 100 ng/ml GM-CSF (CD34-mDC). Cells and supernatants were analyzed after 48 hours.

Allogeneic mixed lymphocyte reaction (MLR)
Responder T cells were isolated from a buffy coat. The mononuclear fraction was incubated with anti-CD15- and anti-CD235-coated microbeads (Miltenyi Biotech GmBH) and PE-labeled antibodies against CD1c (AD5-8E7, Miltenyi Biotech GmBH), CD14, CD19 (J4.119, Beckman Coulter, Woerden, The Netherlands), CD56 (MY31, BD Biosciences) and CD123 (SSDCLY107D2, Beckman Coulter), followed by incubation with anti-PE-coated microbeads (Miltenyi Biotech GmBH). T cells were isolated through

negative selection according to manufacturer's instructions (Miltenyi Biotech GmBH). Irradiated DCs (mo-DC 40 Gy; CD34-mDC 30 Gy) were added in graded doses to 15 × 10^4 (mo-DC) or 2 x 10^4 (CD34-mDC) allogeneic T cells in 96-well round bottom plates in RPMI-1640 containing 8% heat inactivated FBS. Proliferation was quantified by incubation with 1 µCi (37 kBq) [methyl-^{3}H]thymidine (NEN™ Life Science Products, Inc., Boston, MA) during the last 18 hours of 6-day cultures.

Cell phenotype analysis and apoptosis detection by flow cytometry
Surface markers: For phenotypic analysis, cells were washed in PBS containing 1% bovine serum albumin (BSA), 1% heat inactivated human serum and 0.02% NaN_3. Labeling of cell surface markers was performed on ice, using fluorochrome-conjugated antibodies against the following Ag: CD1a, CD14, Langerin/CD207 (DCGM4, Beckman Coulter), DC-SIGN, (DCN46, BD Biosciences), CD40 (mAB89, Beckman Coulter), CD83 (HB15e, BD Biosciences), CD86 (Fun-1, BD Biosciences), B7-H1 (biotin-conjugated; MIH1, eBioscience), HLA-DR (L243, BD Biosciences) and CCR7 (150503, R&D systems). Binding of biotin-conjugated antibodies was visualized by a second incubation with fluorochrome-conjugated streptavidin (BD Biosciences). *Apoptosis:* Apoptosis was detected by determination of phosphatidyl serine exposure and membrane permeability. Cells were harvested, washed in Annexin buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), incubated with Annexin V-FITC (BD Biosciences) for 30 minutes on ice and subsequently taken up in 1 µg/ml Propidium Iodide (PI; Sigma-Aldrich). *Mitochondrial transmembrane potential:* Mitochondrial dysfunction was assessed using rhodamine 123 (Calbiochem). Cells were incubated in RPMI-1640 supplemented with 8% heat inactivated FBS and penicillin/streptomycin at 37°C for 30 minutes in the presence of 0.1 µg/ml rhodamine 123. Then, cells were washed and taken up in PBS. *Caspase-3 activation:* For intracellular staining of active caspase-3, cells were fixed (15 minutes) and subsequently permeabilized (5 minutes) by incubation in fixation medium and permeabilization medium (Fix&Perm, ADG Bio Research GmBH, Kaumberg, Austria). Labeling of cleaved caspase-3 was performed on ice by 15 minutes incubation with FITC-conjugated rabbit-anti-active caspase-3 (C92-605; BD Biosciences) in permeabilization medium. Rabbit serum was added to block aspecific labeling. *Assessment:* Assessment was performed using a FACSCalibur or FACSCanto II (BD Biosciences) and data were analyzed using FlowJo software (www.flowjo.com).

Detection of cytokine production by ELISA
The commercially available ELISA kits for human IL-6, IL-10, IL-12p70 and IFN-γ (eBioscience) were used according to manufacturer's instructions. The detection limits of these assays were 2 pg/ml (IL-6 and IL-10) and 4 pg/ml (IL-12p70 and IFN-γ).

Western blot analysis
Cells were washed in PBS and lysed in NTEP-lysisbuffer containing 50 mM Tris (pH 7.9), 150 mM NaCl, 5 mM EDTA and 0.5% NP40 supplemented with 1% Protease Inhibitor Cocktail (Sigma-Aldrich). Protein concentrations were determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL). Equal amounts of total lysate (20 µg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to a PVDF membrane (Millipore, Bedford, MA) and incubated with blocking buffer (PBS-0.1%Tween-5%non-fat dry milk-1%BSA) before probing with antibody against IκBα (sc-371, Santa Cruz Biotechnology, Heidelberg, Germany). After subsequent incubation with HRP-conjugated swine-anti-rabbit antibody (DAKO, Glostrup, Denmark) and supersignal (Pierce), blots were exposed to Hyperfilm™ films (Amersham Pharmacia Biotech, UK) to visualize labeled protein. Equal loading was confirmed by coomassie blue staining.

Results

Canonical NF-κB activity regulates differentiation during mo-DC development

The role of canonical NF-κB in human myeloid DC development was first investigated in myeloid DC generated from monocytes. To investigate the importance of canonical NF-κB transcriptional activity, its activation was inhibited by means of the pharmacological inhibitors CAPE, BAY or AS. Effective blocking of NF-κB activity by these inhibitors was shown by

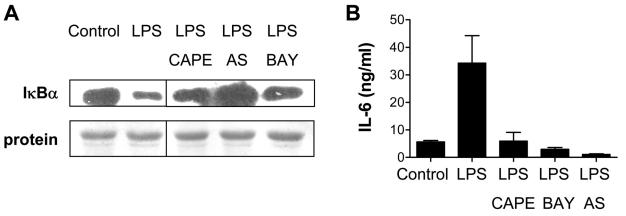

Figure 1. CAPE, BAY and AS inhibit canonical NF-κB activation
Monocytes were cultured in the presence or absence of 100 ng/ml LPS and in the presence or absence of CAPE, BAY or AS. ***A)*** *Total cell lysates were produced after 45 minutes and expression of IκBα was determined. Total protein was stained by coomassie blue to confirm equal loading (n=3).* ***B)*** *Supernatants were harvested after 24 hours. IL-6 concentration was measured by ELISA. Shown are mean±SD of duplicate cultures of a representative experiment (n=4).*

abolished LPS-induced IκBα degradation and IL-6 production (Figure 1). While monocytes cultured with GM-CSF and IL-4 acquired a characteristic DC morphology and phenotype ($CD14^{-}CD1a^{+}DC\text{-}SIGN^{+}$) within 6 days, inhibition of NF-κB by addition of 10 µg/ml CAPE, 1 µM BAY or 1 µM AS during differentiation reduced the CD1a expression by day 6 monocyte-derived cells (Figure 2A,B). This effect was dose-dependently reduced when the inhibitors were used at lower concentrations, while increased concentrations appeared extremely toxic (Figure 2C). The induction of DC-SIGN expression was also affected by the inhibitors, whereas monocyte marker CD14 was downregulated in all conditions and CD40 expression was normal regardless of the presence of NF-κB inhibitors (Figure 2A,B). These data indicate that canonical NF-κB activity is required for complete mo-DC differentiation.

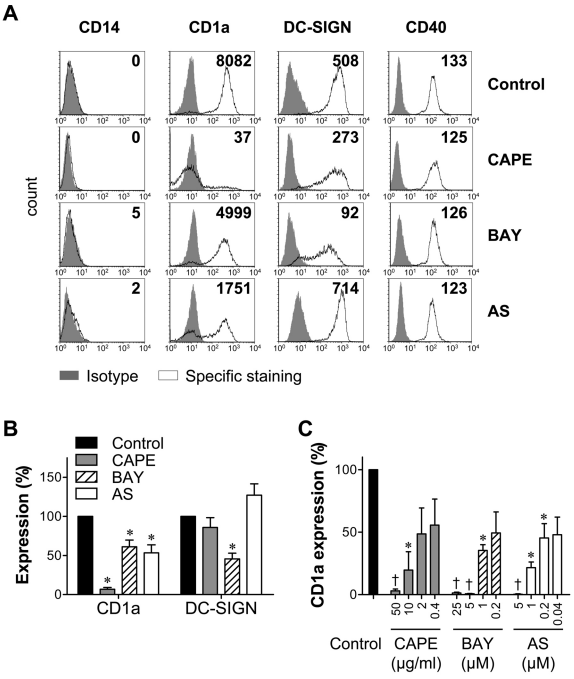

Figure 2. Mo-DC differentiation is dependent on NF-κB
CAPE, BAY, AS or their solvent DMSO was added to mo-DC differentiation cultures at days 0, 2 and 5. ***A)*** *Day 6 cells were harvested and analyzed for the expression of CD14, CD1a, DC-SIGN and CD40. Viable cells were gated on FSC/SSC. FACS plots are representative of at least 11 experiments. Specific staining intensities, calculated by subtracting background fluorescence from mean fluorescence intensity (MFI) of cells stained with specific antibodies, are indicated in the figure.* ***B)*** *CD1a and DC-SIGN specific MFIs were standardized to control. Shown are mean±SEM from at least 11 independent experiments with different donors.* ***C)*** *CAPE, BAY or AS were added in increasing concentrations. Day 6 cells were analyzed for the expression of CD1a, specific MFIs were calculated and standardized to control. Mean±SEM CD1a MFIs are shown (n=3).* † *over 95% cell death; * p<0.05, paired Student t-test*

NF-κB inhibition allows differentiation of functional APC

The functional abilities of the cells generated were investigated next. Cells differentiated in the presence of NF-κB inhibitors demonstrated normal endocytosis, as appeared from the unaffected uptake of $Dextran^{FITC}$ (Figure 3A). Although cells differentiated in the presence of CAPE showed a slightly higher CD86 and HLA-DR expression than control mo-DC at

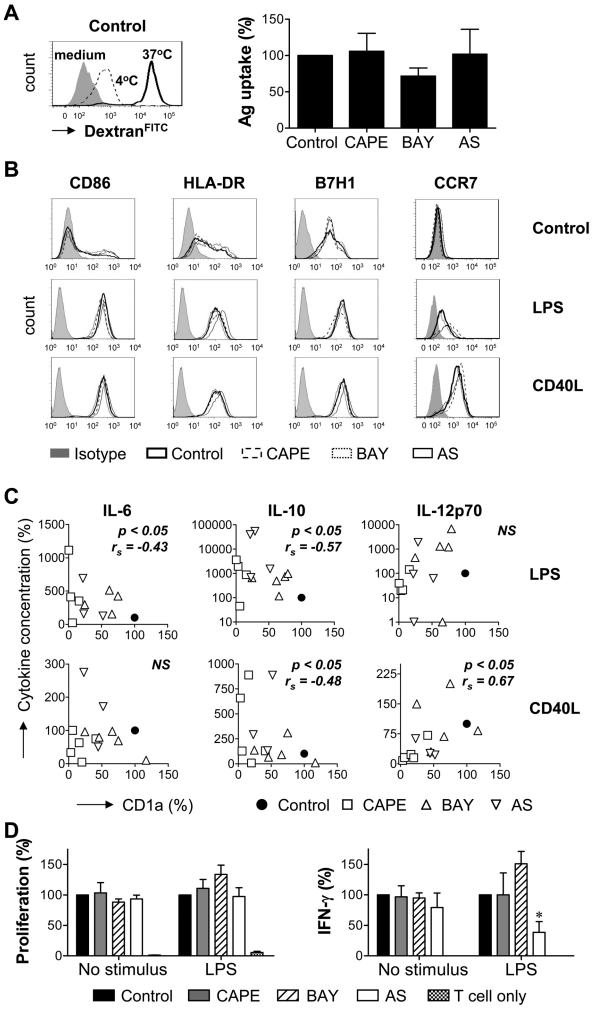

Figure 3. NF-κB inhibition allows differentiation of functional APC

CAPE, BAY, AS or their solvent DMSO was added to mo-DC differentiation cultures at days 0, 2 and 5. ***A)*** *Cells were harvested at day 6, thoroughly washed and incubated with Dextran^{FITC} for 2 hours at 37°C. Surface binding was determined by incubation at 4°C. Ag uptake was calculated as "MFI 37°C – MFI 4°C". Cell debris, as determined by FSC/SSC, was excluded from the analysis. Data represent mean±SEM Ag uptake standardized to control (n=3).* ***B)*** *Day 6 cells were harvested, washed thoroughly and counted with trypan blue exclusion. Equal cell numbers for all conditions were subsequently stimulated with or without 100 ng/ml LPS or L-CD40L. Cells were harvested after 48 hours of stimulation and analyzed for the expression of CD86, HLA-DR, B7H1 and CCR7. Viable cells were gated on FSC/SSC. FACS plots representative of at least 3 experiments are shown.* ***C)*** *Day 6 cells were harvested, analyzed for CD1a expression by flow cytometry, washed thoroughly and counted with trypan blue exclusion. Equal cell numbers for all conditions were subsequently stimulated with or without 100 ng/ml LPS or L-CD40L. Supernatants were harvested after 48 hours of stimulation and IL-6, IL-10 and IL-12p70 concentrations were determined by ELISA. CD1a expression and cytokine concentration in supernatants were standardized to control. Data for all inhibitors were combined, and the spearman's rank correlation coefficient (r_s) was calculated. Significance and r_s are indicated. Data are derived from 3-5 experiments.* ***D)*** *Cells were harvested at day 6, washed thoroughly and used in an allogeneic MLR immediately (n=3) or after 24 hours of stimulation with LPS (n=4). Equal DC:T cell ratio's in all conditions were ensured by counting the cells with trypan blue exclusion just prior to co-incubation. T cell proliferation was quantified by incubating the cells with [methyl-^{3}H] thymidine during the last 18 hours of 6-day cultures. ELISA determined IFN-γ concentration in supernatants harvested at day 5. Proliferation and IFN-γ concentrations were standardized to control. Shown are mean±SEM proliferation in cultures containing DC and T cells in a 1:30 ratio and mean±SEM IFN-γ concentrations from cultures containing DC and T cells in a 1:10 ratio. * p<0.05, paired Student t-test*

the end of differentiation (day 6), this difference was lost after two additional days of culture in the absence of inhibitors, resulting in similar costimulatory molecule expression in all conditions (Figure 3B and data not shown). LPS- or CD40L-stimulation in the absence of inhibitors induced upregulation of all costimulatory molecules, regardless of the presence of NF-κB inhibitors during differentiation (Figure 3B). In addition, CCR7 expression could be induced in all conditions (Figure 3B). When comparing LPS- and CD40L-induced cytokine production by cells differentiated in the presence of any of the three NF-κB inhibitors to cytokine secretion by control mo-DC, no significant changes were observed due to high inter-donor variability (Figure 3C). Interestingly however, when cells generated with different NF-κB inhibitors were analyzed together and the inhibitor-induced alterations in CD1a expression, as typical read-out for phenotypic differentiation, were combined with the induced modifications of IL-6, IL-10 or IL-12p70 production, a rather weak but significant correlation was found (Figure 3C). Although these data suggest that, next to a clear effect on

phenotype, differentiation of mo-DC in the presence of NF-κB inhibitors might also modestly affect their LPS- and CD40L-induced cytokine production, the T cell stimulatory capacity of these cells was not significantly different from mo-DC generated under control conditions. Both control and LPS-stimulated cells from all differentiation conditions induced similar T cell proliferation in allogeneic MLR (Figure 3D). Although IFN-γ production by T cells co-cultured with LPS-stimulated AS-differentiated cells was reduced compared to the control, overall no changes in IFN-γ concentrations were observed (Figure 3D). Thus, while phenotypic differences exist between mo-DC differentiated in the presence or absence of NF-κB activity, NF-κB inhibition does not prevent the generation of functional APC.

NF-κB inhibition results in apoptosis

Next to the effects on differentiation, NF-κB inhibition during mo-DC development also resulted in reduced cell yields at day 6 (Figure 4A), indicating a potential role in the regulation of survival. To investigate this further, mo-DC differentiation cultures were harvested at various time points and analyzed for apoptosis. Induction of apoptosis was clearly shown by increased Annexin V staining of the PI^- population in inhibitor cultures compared to control at days 2, day 3 and day 6 ($p<0.05$) (Figure 4B and data not shown). In addition, activation of caspase-3 and reduced mitochondrial transmembrane potential was observed at all time points (Figure 4C,D and data not shown). Together, these data show that NF-κB activation during mo-DC development is required to avoid the induction of apoptosis and ensure cell survival.

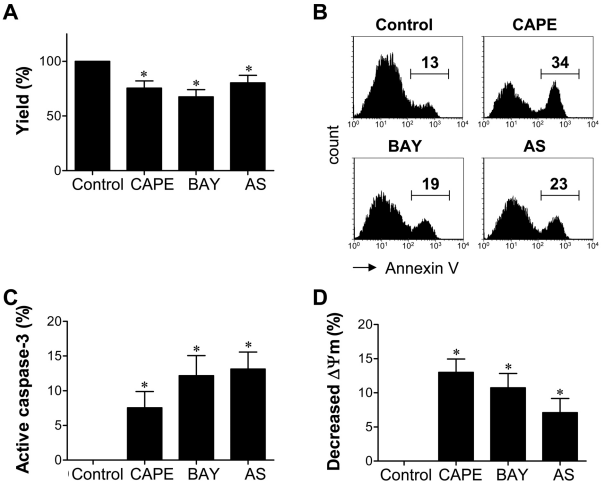

Figure 4. Apoptosis induction upon NF-κB inhibition

CAPE, BAY, AS or DMSO was added to mo-DC differentiation cultures at days 0, 2 and 5. ***A)*** *At day 6, cells were counted with trypan blue exclusion. Yields were standardized to control cultures. Data shown represent mean±SEM of at least 10 independent experiments with different donors.* ***B)*** *Cells were harvested at day 2 and analyzed for AnnexinV/PI staining. PI^- cells were gated. Representative FACS plots are shown (n=4).* ***C)*** *Day 2 cells were analyzed for active caspase-3. Data were standardized to control by subtracting the percentage of positive cells in the control culture from the percentage of positive cells in inhibitor cultures. Mean±SEM percentages of cells showing active caspase-3 are shown (n=4).* ***D)*** *Day 2 cells were analyzed for mitochondrial transmembrane potential ($\Delta\Psi_m$). The percentage of cells with a decreased $\Delta\Psi_m$ was determined and data were standardized to control as in (C). Shown are mean±SEM (n=4). * $p<0.05$, paired Student t-test*

CD34-derived myeloid DC development requires canonical NF-κB

Next, we investigated the importance of NF-κB in the development of other myeloid DC types, the CD34-derived intDC and LC. Six days of culture in the presence of CAPE resulted in a large reduction in cell yield (98±2%) (Figure 5A). Within the viable cells, CAPE abrogated the generation of $CD14^+CD1a^-$ pre-intDC from $CD34^+$ HPC, whereas the development of $CD14^-CD1a^+$ pre-LC was relatively retained as observed by flow cytometry 4 and 6 days after the start of culture (Figure 5B,C). The combination of reduced yield and inhibited differentiation resulted in a strong dose-dependent reduction in pre-LC and pre-intDC numbers upon exposure to CAPE (Figure 5D). These data show an important role for NF-κB in CD34-derived pre-DC development.

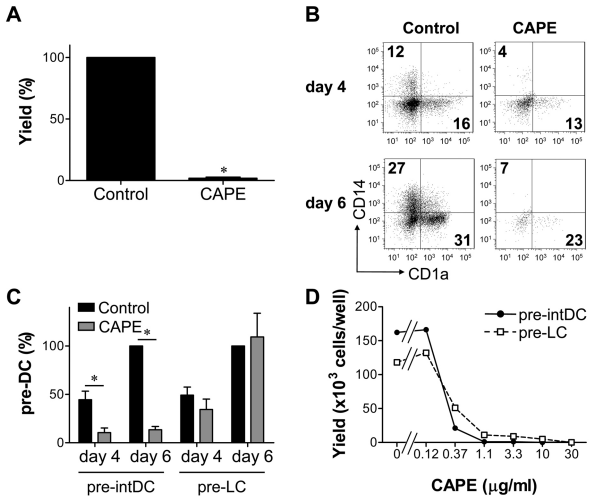

Figure 5. NF-κB is required for CD34-derived pre-DC development

CAPE or its solvent DMSO was added to CD34-derived myeloid DC differentiation cultures from day 0. ***A)*** *Cells were harvested at day 6 and counted with trypan blue exclusion. Yields were standardized to control cultures. Data shown represent mean±SEM of 3 independent experiments with different donors.* ***BC)*** *Cells were harvested at days 4 and 6 and analyzed for the expression of CD1a and CD14. Viable cells were gated on FSC/SSC. Percentages $CD14^+CD1a^-$ pre-intDC and $CD14^-CD1a^+$ pre-LC were determined and standardized to the control at day 6. Representative FACS plots (B) and mean±SEM percentage pre-DC (C) are shown (n=5).* ***D)*** *CAPE was added in increasing concentrations. Cells were harvested at day 6, counted with trypan blue exclusion and analyzed for the expression of CD1a and CD14. Yields of $CD14^+CD1a^-$ pre-intDC and $CD14^-CD1a^+$ pre-LC were calculated. * p<0.05, paired Student t-test*

Next, differentiation of pre-DC into terminally differentiated DC was investigated. CAPE was added to FACSorted pre-intDC or pre-LC to separately examine the two individual subsets. Under control conditions, $CD1a^-CD14^+$ pre-intDC differentiated to $CD1a^+CD14^-$ intDC via an intermediate $CD1a^+CD14^+$ state, which was clearly present 48 hours after pre-intDC isolation. In the presence of CAPE however, $CD1a^+$ cells did not appear ($p<0.01$) (Figure 6A). 48 hour cultures of pre-LC resulted in more than 90% $CD1a^+$ cells in the presence as well as in the absence of CAPE (Figure 6A). These data suggest that NF-κB activity is required for intDC but not for LC differentiation. However, a significant reduction in viable cells was observed following CAPE-treatment of both subsets (Figure 6B). Already within 24 hours, a 50±9% and 54±9% loss of viable cells was observed in intDC and LC cultures respectively. In the next days further cell loss became apparent, resulting in only

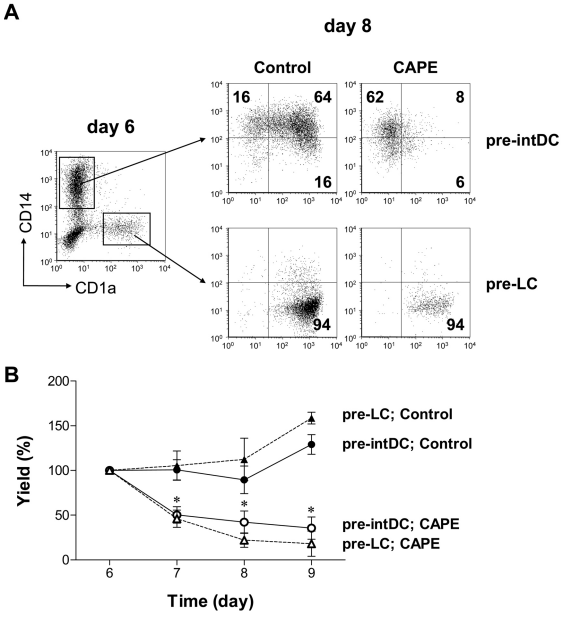

Figure 6. Survival and differentiation of pre-DC are dependent on NF-κB

CAPE or its solvent DMSO was added to day 6 FACSorted pre-intDC and pre-LC. Cells were harvested at day 7, 8 or 9, counted with trypan blue exclusion and analyzed for the expression of CD1a and CD14. ***A)*** *Viable cells were gated on FSC/SSC. Shown are representative FACS plots from day 8 cultures (n=3).* ***B)*** *Yields were standardized to the number of cells at start of culture (day 6). Mean±SEM of at least 3 experiments are shown. * p<0.05, paired Student t-test*

36±12% and 18±14% cells remaining after 3 days of treatment. Together, these data show that canonical NF-κB regulates differentiation as well as cell numbers during CD34-derived myeloid DC development.

Induction of apoptosis during CD34-derived myeloid DC development

As opposed to mo-DC cultures that lack proliferation, the observed cell loss in NF-κB-inhibited CD34-derived myeloid DC cultures could result from inhibition of proliferation as well as induction of apoptosis. To investigate the role of apoptosis, NF-κB inhibitors CAPE or AS were added to CD34-myeloid DC cultures at various time points. As shown by the increased Annexin V binding ($p<0.05$), apoptosis was induced upon NF-κB inhibition during both pre-DC development and terminal DC differentiation (Figure 7A,B). In addition, increased caspase-3 activation and loss of mitochondrial transmembrane potential was observed (Figure 7C,D). Thus, the observed reduction of cells upon NF-κB inhibition can at least partly be explained by the induction of apoptosis.

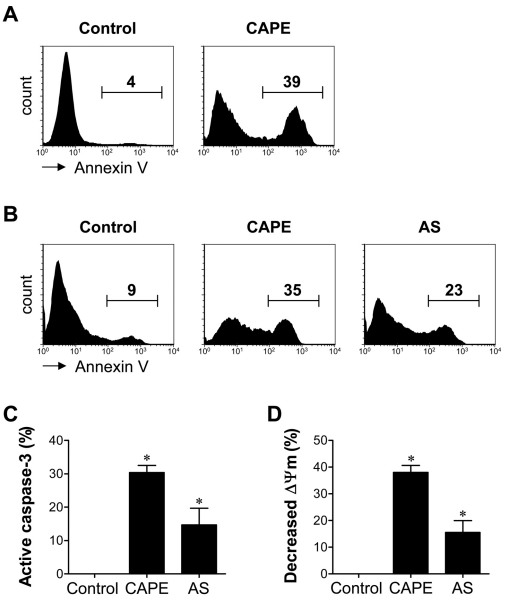

Figure 7. Inhibition of NF-κB leads to apoptosis in developing CD34-derived myeloid DC

A) *CAPE or DMSO was added to CD34-derived myeloid DC differentiation cultures at day 0. Cells were harvested at day 4 and analyzed for apoptosis by Annexin V and PI staining. Representative FACS plots of PI- cells are shown (n=3).* ***B-D)*** *CAPE, AS or DMSO was added to day 6 pre-DC. Cells were harvested at day 8 and analyzed for AnnexinV/PI staining, active caspase-3 and $\Delta\Psi_m$. Shown are FACS plots showing Annexin V staining of PI- cells (B), mean±SEM percentages of cells showing active caspase-3, standardized to control (C) and mean±SEM percentages of cells with a decreased $\Delta\Psi_m$, standardized to control (D) (n=5). Data were standardized to control by subtracting the percentage of positive cells in the control culture from the percentage of positive cells in inhibitor cultures. * p<0.05, paired Student t-test*

Myeloid DC survival and function requires canonical NF-κB

Although we showed that NF-κB activity is required for survival and phenotypic differentiation of myeloid DC, NF-κB inhibition during differentiation hardly affected the function of the APC generated. To investigate whether NF-κB inhibition after terminal differentiation affects DC survival or function, CAPE or AS were added to fully differentiated mo-DC, CD34-intDC and CD34-LC. Apoptosis was induced upon 24- or 48-hour culture in the presence of inhibitors ($p<0.05$) (Figure 8A), demonstrating that also differentiated myeloid DC require NF-κB activation to ensure their survival. Mo-DC, CD34-LC and CD34-intDC could be rescued from inhibitor-induced apoptosis by stimulation with CD40L, while LPS only prevented death of mo-DC and CD34-LC (data not shown). Both LPS- and CD40L-stimulation induced maximal expression of CD83, CD86 and HLA-DR in all cell types, independent of the presence or absence of NF-κB inhibitors (Figure 8B and data not shown). Also B7H1, which was only expressed by mo-DC, was similarly expressed in all conditions. While these data show that NF-κB is redundant for the induction of costimulatory molecule expression, NF-κB inhibition

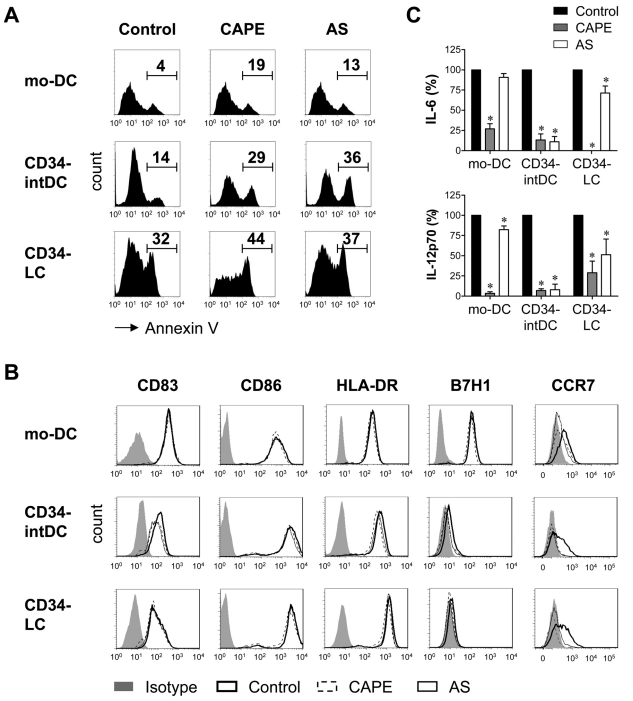

Figure 8. NF-κB is redundant for induction of costimulatory molecules, but not for induction of CCR7 expression or cytokine production

Mo-DC were differentiated under control conditions until day 6. Control CD34-derived pre-intDC and pre-LC were isolated by FACSort at day 6 and further cultured under control conditions until day 10 (LC) or day 12 (intDC). Terminally differentiated DC were cultured with or without CD40L-transfected L-cells in the presence of DMSO, CAPE or AS. ***A)*** *Cells cultured in the absence of CD40L were harvested after 24 (mo-DC) or 48 (CD34-DC) hours and analyzed for AnnexinV/PI staining. FACS plots of PI- cells are representative of at least 3 experiments.* ***B)*** *CD40L-stimulated cells were harvested after 48 hours and analyzed for the expression of CD83, CD86, HLA-DR, B7H1 and CCR7. Viable cells were gated on FSC/SSC. Representative FACS plots are shown (n=3).* ***C)*** *Supernatants of CD40L-stimulated cells were harvested after 48 hours. IL-6 and IL-12p70 concentrations were determined by ELISA and standardized to control. Mean±SEM standardized cytokine concentrations of at least 3 independent experiments with different donors are shown. * p<0.05, paired Student t-test*

almost completely abrogated the induction of CCR7 expression by mo-DC as well as CD34-DC ($p<0.05$) (Figure 8B), indicating that canonical NF-κB may be involved in the regulation of lymph node migration. Furthermore, inhibition of NF-κB activity significantly reduced 24 and 48 hour activation-induced IL-6 and IL-12p70 secretion by all DC types (Figure 8C and data not shown). In addition, T cell stimulatory capacity of myeloid DC that were incubated with inhibitors prior to use in MLR was reduced compared to control DC. Although no difference in T cell proliferation could be detected, the production of IFN-γ was reduced in MLR using DC pre-treated with CAPE or AS (Figure 9). A similar pattern was observed at earlier time points (data not shown), indicating that the reduced IFN-γ concentrations resulted from reduced secretion rather than a delayed induction of T cell proliferation. Thus, canonical NF-κB activity regulates functional maturation and thereby T cell stimulatory capacity of both mo-DC and CD34-derived myeloid DC.

Discussion

DC are crucial in the induction of immunity as well as tolerance. Their development, survival and maturation all critically influence the regulation of immune responses. While mouse knock-out studies have shown a role for the canonical NF-κB pathway in murine myeloid DC biology, a detailed analysis of its function in the different processes of functional human DC development is lacking. The present study investigated the role of canonical NF-κB activity in the development and function of three different human myeloid DC subsets. By using several distinct pharmacological inhibitors the risk of observing non-NF-κB related side effects was minimized. Overall, only minor differences between the inhibitors were apparent. Canonical NF-κB activity was found to regulate differentiation, survival, maturation

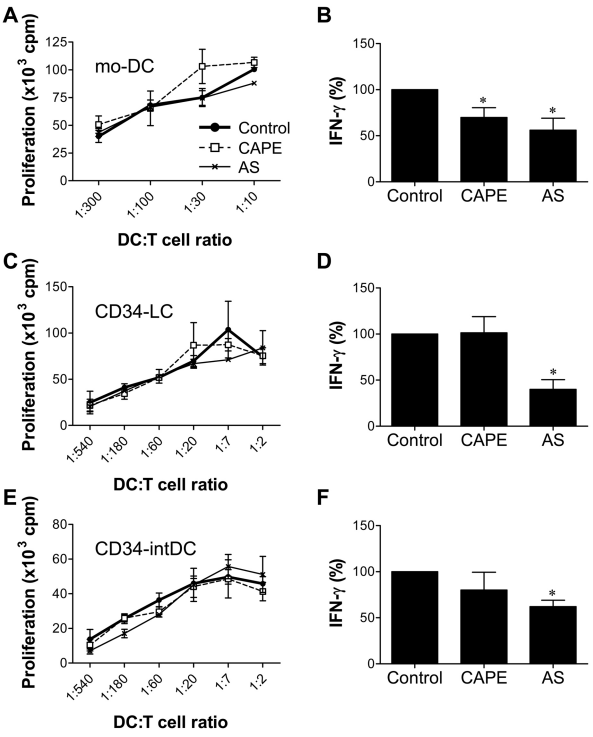

Figure 9. NF-κB inhibition reduces T cell stimulatory capacity

*Mo-DC (AB) were differentiated under control conditions until day 6 (n=5). Control CD34-derived pre-intDC and pre-LC were isolated by FACSort at day 6 and further cultured under control conditions until day 10 (LC) (CD) or day 12 (intDC) (EF) (n=3). Terminally differentiated DC were cultured with 100 ng/ml LPS in the presence of DMSO, CAPE or AS. Cells were harvested after 24 hours, washed thoroughly and used in an allogeneic MLR. Equal DC:T cell ratio's in all conditions were ensured by counting the cells with trypan blue exclusion just prior to co-incubation. T cell proliferation was quantified by incubating the cells with [methyl-^{3}H]thymidine during the last 18 hours of 6-day cultures. Shown are mean±SD of triplicate cultures of a representative experiment (A,C,E). ELISA determined IFN-γ concentration in supernatants from the highest DC:T cell ratio, harvested at day 5. Concentrations were standardized to control. Mean±SEM concentrations are shown (B,D,F). * p<0.05, paired Student t-test*

and function of mo-DC as well as CD34-mDC.

In mice, simultaneous deficiency of p50 and RelA has been shown to result in DC loss, but whether this was caused by inhibited differentiation or survival remained unclear (23). Canonical NF-κB plays a crucial role in anti-apoptosis by the induction of anti-apoptotic genes (41). Accordingly, its activity has been associated with augmented cell survival in various cell types (42-45). In line, here we show that during development of human myeloid DC, either from monocytes or from CD34$^+$ HPC, cell survival is strongly dependent on intact canonical NF-κB activation. Also survival of terminally differentiated mo-DC and CD34-mDC required NF-κB activity, although the cells could be rescued from inhibitor-induced apoptosis by the simultaneous presence of activation signals. Reduced survival due to decreased NF-κB activity has been described even in the presence of activation stimuli (23, 30, 46-47), but these discrepancies probably result from differences in species, DC type or experimental set-up. Together, our data clearly show that canonical NF-κB plays a nonredundant role in the regulation of survival of differentiating as well as fully differentiated monocyte- and CD34-derived human myeloid DC.

While apoptosis is traditionally thought to require caspase activation, caspase-independent apoptosis has also been described (48). In the present study, NF-κB inhibition-induced death was associated with phosphatidyl serine exposure, an archetypal caspase-dependent event (48-49), and caspase-3 activation. The additional loss of mitochondrial transmembrane potential further implies a role for mitochondria in the induction and/or amplification of NF-κB inhibition-induced apoptosis. This is in accordance with the role of NF-κB in the induction of genes opposing caspase activation (50-52) as well as the transcriptional control of Bcl-2 family members (42, 53-55), whose altered balance can lead to pore formation in the mitochondrial outer membrane, resulting in the release of apoptosis-promoting mitochondrial intermembrane space proteins (56).

As shown here, not only survival but also differentiation of mo-DC and CD34-derived intDC depends on canonical NF-κB activity. Inhibited differentiation of CD34-derived intDC upon NF-κB inhibition was demonstrated by the abrogated development of pre-intDC and the inhibited CD1a acquisition during terminal differentiation. Similarly, the induction of CD1a and DC-SIGN expression on mo-DC was reduced by specific NF-κB inhibitors, while loss of the monocyte marker CD14 occurred regardless of the presence of inhibitors. Reduced expression of differentiation markers was observed even when inhibitor-induced apoptosis was blocked by simultaneous addition of the pan-caspase inhibitor ZVAD-fmk (data not shown), indicating that the reduced differentiation was not merely a side effect of the presence of dying cells or debris. Furthermore, apoptosis-induction in developing myeloid DC is not necessarily associated with inhibited differentiation, as demonstrated in studies using inhibitors of unrelated pathways that affect survival but not differentiation of the developing DC (39, 57). Thus, canonical NF-κB activation appears to be nonredundant in the differentiation of human myeloid DC.

In spite of their altered phenotype, cells differentiated under NF-κB inhibition showed normal Ag uptake and normal maturation upon TLR triggering or stimulation with T cell derived signals such as CD40L. Functional analysis of CD34-derived myeloid DC generated under NF-κB inhibition could not be performed due to massive cell loss, but for mo-DC, Ag uptake capacity and induction of costimulatory molecules and CCR7 was similar for cells differentiated in the presence or absence of canonical NF-κB activity. When focusing on a single NF-κB inhibitor, cytokine production by mo-DC generated in the presence of CAPE, BAY or AS was not significantly different from control mo-DC. Nevertheless, when combining all experiments and NF-κB inhibitors, a shift in cytokine profile partially related to inhibitor-induced phenotypic alterations could be observed. Although both the reduced expression of CD1a and DC-SIGN, molecules involved in Ag presentation and T cell stimulation (58-59), and the altered cytokine production profile could influence the induction of T cell responses, no significant changes in allogeneic T cell stimulatory capacity were found. The unchanged T cell stimulatory capacity of mo-DC generated with CAPE, BAY or AS seems to be in contrast to the reported tolerogenic role of BAY (28). However, BAY only induced tolerance when it was used at a concentration of 2.5 μM or higher. At these concentrations, but not at the 1 μM concentration used in the present study, BAY also inhibited the noncanonical NF-κB protein RelB (28), a protein of crucial importance for DC immunogenicity. Thus, while noncanonical NF-κB is essential for the development of functional DC, we show here that despite the role of canonical NF-κB proteins in the regulation of survival and phenotypic differentiation, the function of DC differentiated in the presence of canonical NF-κB inhibitors was similar to control DC.

As discussed above, canonical NF-κB inhibition during DC differentiation hardly affected the ability to respond to activation-inducing stimuli. The presence of canonical NF-κB inhibitors during activation of mo-DC or CD34-mDC however, clearly inhibited DC function. In addition to previous studies on mouse myeloid DC and human mo-DC (30-31, 60-63), the present study demonstrates that not only mo-DC but also CD34-derived intDC and LC show reduced secretion of pro-inflammatory cytokines IL-6 and IL-12p70 as well as reduced T cell stimulatory capacity upon NF-κB inhibition during activation. The reduced activation-induced CCR7 expression in the presence of NF-κB inhibition could further abrogate DC functionality, as this could affect lymph node migration and hence interaction with T cells. As DC survival was unaffected in these cultures (data not shown), the reduced functionality reflects regulation of DC function by NF-κB, rather than side effects of cell death or reduced cell numbers. Ag uptake capacity was not affected by inhibition of NF-κB (data not shown). The unchanged upregulation of CD83, CD86, HLA-DR and B7H1 shown here seems contradictory to some studies describing a role of canonical NF-κB in activation-induced

upregulation of costimulatory molecules (30, 61, 64), but is in accordance with others (28, 31). These discrepancies could result from differences in experimental set-up. It is however not unlikely that the canonical NF-κB pathway contributes to the induction of cytokine secretion, costimulatory molecule expression and chemokine receptor expression to a different extent. Our data suggest that while functional maturation and CCR7 expression of human myeloid DC is regulated by canonical NF-κB activation, this pathway is redundant for the upregulation of costimulatory molecules.

Several immunomodulatory compounds such as corticosteroids, vitamin D_3 and IL-10 have been reported to inhibit human myeloid DC development (36, 65-70). In contrast to specific canonical NF-κB inhibitors, addition of these compounds during DC differentiation not only affects the survival and phenotype of the generated cells, but also affects functional differentiation yielding tolerogenic rather than immunogenic DC (68, 70-71). Also in direct comparison in the same experiment, dexamethasone, vitamin D_3 and IL-10 affected mo-DC functional differentiation much more vigorously than CAPE, BAY or AS (van de Laar, unpublished data). These data challenge the suggestion that the immunosuppressive function of corticosteroids, vitamin D_3 and IL-10 results from modulation of canonical NF-κB activity alone.

Under both steady state and inflammatory circumstances, DC are continuously being replenished from different precursors, including monocytes as well as pre-DC (4, 6). Manipulation of canonical NF-κB activity by drugs, pathogens or tumors trying to escape immunity will affect development of myeloid DC from both monocytic and pre-DC precursors, although CD34-derived myeloid DC development seemed to depend even more strongly on intact NF-κB activity. Furthermore, the functionality of differentiated DC will be affected by interference with NF-κB activity. The importance of NF-κB in all developmental phases of all investigated myeloid DC types implies that inhibition of canonical NF-κB will, at any moment, interfere with DC-induced immune activation, due to reduced DC numbers as well as the inhibition of activation-induced DC functionality. This knowledge might provide an explanation for the strategies used by certain tumors and pathogens to avoid immune recognition and might open up new tools for the invention of better and more specific immune therapies aiming at either enhancing or suppressing immunity.

In conclusion, canonical NF-κB activation is of crucial importance for the development and function of monocyte- as well as CD34-derived human myeloid DC. Our data not only confirm previous studies suggesting that canonical NF-κB is involved in mo-DC function, but also show the importance of its activation for the functionality of other human myeloid DC subtypes. Additionally, we show that canonical NF-κB is pivotal for differentiation and survival of human mo-DC, CD34-intDC and CD34-LC. This fundamental insight into the regulation of DC development and function may help in understanding how tumors and/or pathogens escape immunity and will contribute to the development of novel treatment strategies aiming to interfere with the human immune system.

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5

TIGHT CONTROL OF STAT5 ACTIVITY DETERMINES HUMAN CD34-DERIVED INTERSTITIAL DENDRITIC CELL AND LANGERHANS CELL DEVELOPMENT

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Abstract

Despite the crucial function of dendritic cells (DC) in immunity, the molecular mechanisms regulating human DC development remain poorly defined. STAT5 regulates various hematopoietic lineages and is activated by GM-CSF, a critical cytokine in DC development. Here, we investigated the role of STAT5 during differentiation of human $CD34^+$ hematopoietic progenitors into precursor DC (pre-DC) and their subsequent differentiation towards interstitial DC (intDC) and Langerhans cells (LC). Inhibiting STAT5 activity by dominant-negative STAT5 promoted LC commitment of hematopoietic progenitors, but resulted in loss of pre-intDC development, showing subset-specific regulation. Increasing the low endogenous STAT5 activity by ectopic STAT5 activation downregulated expression of the critical DC transcription factor PU.1 and abrogated commitment to either DC lineage. In contrast, high STAT5 activity was beneficial in already committed pre-DC: terminal DC differentiation was associated with increased endogenous STAT5 phosphorylation levels, JAK2-STAT5 inhibition reduced terminal DC differentiation, and conditional STAT5 activation in pre-DC improved development of BDCA-1^+, DC-$SIGN^+$ and $Langerin^+$ DC with normal maturation and T cell stimulation. These data show that STAT5 critically regulates human DC development, with specific requirements for the level of STAT5 activation at distinct differentiation stages. By regulating STAT5 activity, cytokines present at specific locations and under different pathophysiological conditions can determine the fate of DC precursors.

Introduction

Although dendritic cells (DC) represent only a very small fraction of the hematopoietic system, their specialized functions are crucial for both the induction of immunity and the maintenance of tolerance (1). Multiple DC subtypes can be recognized based on surface markers, localization, functional abilities and ontogeny, but the identified subsets do not completely overlap between the different species (2-5). The human myeloid DC subsets, including interstitial DC (intDC) and Langerhans cells (LC), are related to conventional DC identified in mice (2, 6).

Being relatively short-lived, DC are continuously replenished from organ-resident or blood-derived precursors (7). Direct precursor DC (pre-DC) as well as immune cells with their own functionality but able to generate DC, such as monocytes, constantly develop from early hematopoietic progenitors in a complex cytokine-driven process including several developmental stages at different anatomic locations (2, 7-11). Since the cytokine milieu varies between anatomic locations, the intracellular signaling cascades required for DC development may differ between the separate developmental stages. Furthermore, different cytokines and probably also signaling pathways are induced under various pathophysiological conditions. Despite their important role in the regulation of immunity, the molecular mechanisms responsible for the development of human DC are largely unknown.

Signal transducer and activator of transcription (STAT)5 plays an important role in hematopoiesis, regulating self-renewal, proliferation and lineage decisions of hematopoietic stem cells and hematopoietic progenitor cells (HPC) (12-16). Most myeloid cells express cytokine receptors that are able to induce STAT5 activation and STAT5 has been shown to regulate the development of various myeloid lineages (13, 17). GM-CSF is one of the cytokines triggering STAT5 phosphorylation via activation of janus kinase 2 (JAK2) (18). Initially the role of GM-CSF for DC development in the steady state was thought to be limited (19-20), but it was recently shown that next to Flt3 ligand also GM-CSF is required for steady state DC development (21). In addition, GM-CSF is thought to play an important role in DC differentiation during inflammation (5, 7).

In mice, GM-CSF-induced STAT5 activation has been shown to inhibit plasmacytoid DC development (22). However, in spite of the importance of STAT5 in hematopoiesis and myeloid lineage development, the role of STAT5 in human myeloid DC development remains unclear. The present study demonstrates that development of human myeloid DC subsets is critically regulated by STAT5 activity. Rather than acting in an on-off binary manner, STAT5 activity needs to be tightly regulated for optimal differentiation of intDC and LC from human $CD34^+$ hematopoietic progenitors. In addition to the level, the timing of STAT5 activation appeared essential. Depending on the developmental stage, manipulation of STAT5 activity resulted in lineage skewing, inhibition or enhancement of DC differentiation. These data indicate that regulation of STAT5 activation represents a mechanism by which cytokines determine the fate of DC precursors. Insight in the molecular mechanisms regulating human DC development will improve the understanding of how DC replenishment from their hematopoietic progenitors is regulated and homeostasis of these important immune cells is maintained.

Materials and Methods

Generation of CD34-derived myeloid dendritic cells

Umbilical cord blood samples were obtained ex-uterine according to legal guidelines. $CD34^+$ hematopoietic progenitor cells were isolated and differentiated towards intDC and LC as described

previously (23-24). Briefly, CD34$^+$ cells, isolated through positive selection using anti-CD34-coated microbeads (Miltenyi Biotec GmBH, Bergish Gladbach, Germany), were cultured in complete medium containing RPMI-1640 (Invitrogen, Breda, The Netherlands), 8% heat inactivated FBS (Sigma-Aldrich, St. Louis, MO), 10 mM Hepes (Invitrogen), 2 mM L-glutamine (Lonza, Breda, The Netherlands), 50 μM β-mercaptoethanol (Merck, Darmstadt, Germany) and penicillin/streptomycin (Invitrogen), supplemented from day 0 till day 7 with 100 ng/ml GM-CSF (Leukine sargramostim, Bayer HealthCare, Seattle, WA), 25 ng/ml stem cell factor (SCF, PeproTech, London, UK), 2.5 ng/ml TNF-α (R&D systems, Abingdon, UK) and 5% heat inactivated AB$^+$ pooled human serum (Lonza). From day 7, only 100 ng/ml GM-CSF was added to complete medium. Where indicated, 1 ng/ml TGF-β (PeproTech), 20 nM 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich), 67 or 20 μM AG490 (Calbiochem, La Jolla, CA) or 100 ng/ml LPS (Invivogen, San Diego, CA) was added to the culture.

Viral transduction of CD34$^+$ cells

Bicistronic retroviral expression vectors (LZRSpBMN) consisting of the gene of interest followed by an internal ribosomal entry site (IRES) in front of the enhanced green fluorescence protein (eGFP) were used. The used vectors contained wild type STAT5a (STAT5a-wt) (17), STAT5a1*6, a constitutively active STAT5a mutant that harbors two point mutations, one in the transactivation domain (S710F) and the other in the DNA-binding domain (H298R) (16), or STAT5aΔ750, a dominant-negative STAT5a mutant that can bind to DNA but is unable to activate transcription (17, 25). A vector expressing only IRES followed by eGFP was used as negative control. The MSCV-STAT5a1*6-ER-IRES-iNGFR (STAT5a1*6-ER) retroviral vector encoding 4-OHT-inducible STAT5a1*6 followed by IRES in front of the truncated neural growth factor receptor (iNGFR) was described before (14). Retrovirus was generated by transient or stable transfection of the retroviral packaging cell line Phoenix-ampho with these DNA constructs (24). CD34$^+$ progenitors were transduced with the viral supernatants in complete RPMI medium supplemented with GM-CSF, TNF-α, SCF and human serum (24).

Analysis of cell surface phenotype, intracellular proteins and apoptosis by flow cytometry

Surface markers: For phenotypic analysis, labeling was performed on ice in PBS containing 1% bovine serum albumin (BSA), 1% heat inactivated human serum and 0.02% NaN_3. Fluorochrome-conjugated antibodies against the following antigens were used: CD1a (HI149, BD Biosciences, Breda, The Netherlands), CD3 (SK7, BD Biosciences), CD11b (ICRF44, BD Biosciences), CD14 (MΦP9, BD Biosciences or TÜK4, Dako, Glostrup, Denmark), CD15 (HI98, BD Biosciences), CD20 (2H7, eBiosciences), CD33 (P67.6, BD Biosciences), CD42b (CLB-MB45, Sanquin, Amsterdam, The Netherlands), CD45 (2D1, BD Biosciences), CD56 (MY31, BD Biosciences), CD61 (VI-PL2, BD Biosciences), CD71 (M-A712, BD Biosciences), CD83 (HB15e, BD Biosciences), CD86 (Fun-1, BD Biosciences), BDCA-1/CD1c (AD5-8E7, Miltenyi Biotec), DC-SIGN/CD209 (DCN46, BD Biosciences), Glycophorin A/CD135a (GPA; GA-R2 (HIR2), BD Biosciences), Langerin/CD207 (DCGM4, Beckman Coulter, Woerden, The Netherlands) and HLA-DR (G46-6, BD Biosciences). *Intracellular:* For analysis of intracellular proteins, cells were fixed (30 minutes) and subsequently permeabilized (15 minutes) by incubation in 2% formaldehyde (Merck) and Perm Buffer III (BD Biosciences). Labeling of phosphorylated or total STAT5 was performed on ice by 30 minutes incubation with antibodies against Y694-phosphorylated STAT5 (rabbit polyclonal, Cell Signaling Technology, Danvers, MA) or total STAT5 (3H7, rabbit monoclonal, Cell Signaling Technology), followed by 30 minutes incubation with biotin-labeled goat-anti-rabbit antibodies (Dako). Labeling was visualized by a third incubation with fluorochrome-conjugated streptavidin (BD Biosciences). *Apoptosis:* Phosphatidyl serine exposure and membrane permeability were analyzed in Annexin buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Cells were incubated with fluorochrome-conjugated Annexin V (BD Biosciences) for 30 minutes on ice and subsequently taken up in 7-aminoactinomycin D (7-AAD, BD Biosciences) or 1 μg/ml Propidium Iodide (PI; Sigma-Aldrich). *Assessment:* Assessment was performed using a FACSCalibur or FACSCanto II (BD Biosciences) and data were analyzed using FlowJo software (www.flowjo.com). Surface markers and intracellular proteins were analyzed on viable cells, gated on FSC/SSC.

Cell isolation by FACSorting

Cells were sorted using a FACSAria SORP (BD Biosciences). Cells transduced with a construct expressing eGFP were isolated based on eGFP expression. STAT5a1*6-ER transduced cells were incubated with a biotin-conjugated antibody against NGFR/CD271 (C40-1457, BD Biosciences), followed by a second incubation with fluorochrome-conjugated streptavidin and fluorochrome-conjugated antibodies against

CD1a and CD14. Labeled cells were life gated on FSC/SSC and sorted into iNGFR$^+$CD14$^+$CD1a$^-$ and iNGFR$^+$CD14$^-$CD1a$^+$ fractions.

Histochemical staining
Cytospins were prepared from 5 x 10^4 cells. After fixation in methanol for three minutes, cytospins were stained in a 50% eosin methylene blue solution according to May-Grünwald (Sigma-Aldrich) for 20 minutes. After rinsing in water for 5 seconds, the nuclei were counterstained with 10% Giemsa solution (Merck) for 15 minutes. Micrographs were acquired at room temperature with an Axioskop 20 microscope (Carl Zeiss, Sliedrecht, The Netherlands) fitted with a 40x/1.3 Plan Neofluor oil objective using Immersol 518N oil (Carl Zeiss), a Nikon Digital Sight DS-U1 camera (Nikon, Lijnden, The Netherlands), and NIS Elements D3.0 image acquisition software (Nikon).

Allogeneic mixed lymphocyte reaction (MLR)
Responder T cells were isolated from a buffy coat through negative selection using PE-labeled antibodies against BDCA-1, CD14, CD19 (J4.119, Beckman Coulter), CD56 (MY31, BD Biosciences and CD123 (SSDCLY107D2, Beckman Coulter), and anti-PE-, anti-CD15- and anti-CD235-coated microbeads (Miltenyi Biotec) (24, 26). Irradiated DC (30 Gy) were added in graded doses to 2 x 10^4 allogeneic T cells in 96-well round bottom plates in RPMI-1640 containing 8% heat inactivated FBS and penicillin/streptomycin. Proliferation was quantified by incubation with 1 µCi (37 kBq) [methyl-^{3}H]thymidine (NEN™ Life Science Products, Inc., Boston, MA) during the last 18 hours of 6-day cultures. To determine IFN-γ concentrations in day 5 supernatants, the commercially available ELISA kit for human IFN-γ (eBioscience, San Diego, CA) was used according to manufacturer's instructions. The detection limit of this assay was 4 pg/ml.

Western blot
Total cell lysates were produced using laemmli sample buffer (0.12 M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 µg/µl bromophenol blue and 35 mM β-mercaptoethanol) and boiled for 5 minutes. Equal amounts of total protein, determined by Lowry method, were separated by 8% SDS-PAGE, transferred to an Immobilon-FL transfer membrane (Millipore, Bedford, MA) and incubated with Odyssey blocking buffer (Westburg, Leusden, The Netherlands) for 30 minutes before probing with antibodies against human PU.1 (rabbit polyclonal, Cell Signaling Technology), Y694-phosphorylated STAT5 (rabbit polyclonal, Cell Signaling Technology), total STAT5 (3H7, rabbit monoclonal, Cell Signaling Technology), β-tubulin (mouse monoclonal, Sigma-Aldrich) or β-actin (C4, mouse monoclonal, Santa Cruz Biotechnology, Heidelberg, Germany). Subsequently, blots were incubated with goat-anti-mouse IgG IRDye 680 (Westburg) or goat-anti-rabbit IgG IRDye 800CW (Westburg). To detect phosphorylated STAT5 (p-STAT5) and total STAT5 on the same blots, expression of p-STAT5 was determined first. The blots were then incubated in strip buffer (1% SDS, 30 mM Tris HCl pH 8.0, 45 mM β-mercaptoethanol) for 8 minutes at 55 °C. After thorough washing, expression of total STAT5 was determined. Blots were scanned using an Odyssey Imager (Li-cor, Lincoln, NE) and analyzed and quantified by Odyssey software (Li-cor).

Results

STAT5 activity is required for intDC differentiation, but redundant for LC commitment
To determine the role of STAT5 in CD34-derived myeloid DC development, we first analyzed the phosphorylation status of STAT5, a measure of STAT5 activation, at different time points during culture. Although STAT5 activation was observed at all time points, increased STAT5 phosphorylation was found at later differentiation stages (Figure 1A,B). To investigate the importance of this transcription factor, STAT5 activity was inhibited by retroviral introduction of STAT5aΔ750, a dominant-negative mutant that contains the Y694 phosphorylation site and can bind to DNA but is unable to activate transcription (25). Ectopic expression of this truncated mutant was confirmed by western blot (Figure 1C).

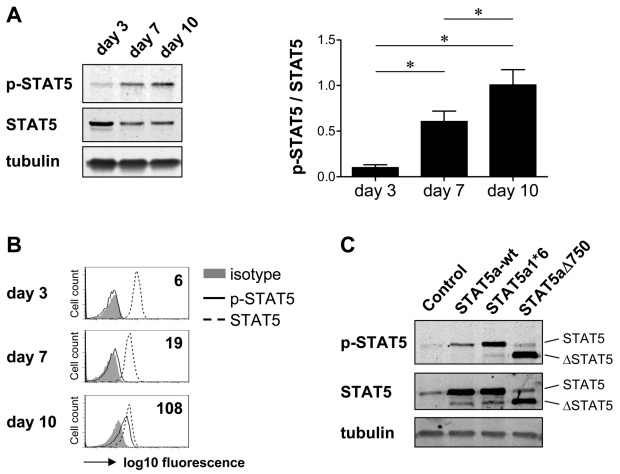

Figure 1. Endogenous STAT5 activity levels increase with CD34-derived myeloid DC differentiation

A) *CD34-derived myeloid DC were differentiated under control conditions. Cells were harvested at days 3, 7 and 10, total cell lysates were prepared and equal amounts of protein were separated on SDS-PAGE. Expression of phosphorylated (p-STAT5) and total STAT5 was determined and the ratio was calculated. Tubulin expression was analyzed to confirm equal loading. A representative experiment and mean±SEM p-STAT5/STAT5 ratios of 4-7 independent experiments with different donors are shown.* ***B)*** *Control cultures were harvested at days 3, 7 and 10. Cells were fixed and intracellular FACS staining for phoshorylated and total STAT5 was performed. For day 10, some experimental variation was apparent. FACS plots representing the majority of 10 independent experiments with different donors are shown. Specific p-STAT5 staining, calculated by subtracting the mean fluorescence intensity of the isotype control from the mean fluorescence intensity measured for p-STAT5, is indicated by the numbers in the FACS plots.* ***C)*** *CD34⁺ HPC were retrovirally transduced with STAT5a-wt, STAT5a1*6, STAT5aΔ750 or a control vector. Transduced cells were isolated by FACSort at day 7. Total cell lysates were prepared and equal amounts of protein were separated on SDS-PAGE. Expression of phosphorylated and total STAT5, as well as tubulin, was determined. A representative experiment of 3 independent experiments with different donors is shown. * p<0.05, paired Student t-test*

CD34⁺ HPC differentiate to CD1a⁺ intDC and CD1a⁺ LC through subset-specific pre-DC that can be distinguished based on their CD14⁺CD1a⁻ and CD14⁻CD1a⁺ phenotypes, respectively (23-24). In contrast to control cultures in which both CD14⁺CD1a⁻ pre-intDC and CD14⁻CD1a⁺ pre-LC were present at day 7, no pre-intDC developed from STAT5aΔ750-transduced HPC (Figure 2A,B). Conversely, the proportion ($p<0.01$) as well as the absolute numbers of pre-LC were increased (Figure 2A,B), indicating that STAT5 activity is redundant or may impede LC commitment. Under control conditions, both terminally differentiated intDC and LC express the myeloid DC marker BDCA-1, whereas only LC acquire Langerin upon terminal differentiation (23-24). Classification of the Langerin⁺ cells as LC was further supported by their specific expression of E-cadherin and cutaneous leukocyte antigen (data not shown). As demonstrated by the increase in Langerin⁺ cells at day 10, STAT5 inhibition induced skewing towards LC (Figure 2C,D). Whereas in control cultures 80% of cells differentiated into BDCA-1⁺ myeloid DC, BDCA-1 was only expressed by 40% of cells derived from STAT5aΔ750-transduced HPC (Figure 2C,D), demonstrating the loss of intDC. Cells derived from STAT5-inhibited cultures had an impaired ability to induce proliferation and IFN-γ production by allogeneic T cells (Figure 2E,F), which could result from reduced DC numbers as well as impaired DC function. The expression of costimulatory molecules by DC from STAT5-inhibited and control cultures was comparable (Figure S1), suggesting intact antigen presenting ability of DC expressing STAT5aΔ750. Thus, whereas CD34-derived intDC development requires STAT5 activity, this transcription factor does not appear to be necessary for commitment to the LC lineage.

Increased STAT5 activity in CD34⁺ HPC inhibits myeloid DC commitment

To further investigate how STAT5 activity influences myeloid DC commitment, CD34⁺ HPC were retrovirally transduced with STAT5a-wt or the constitutively active STAT5a

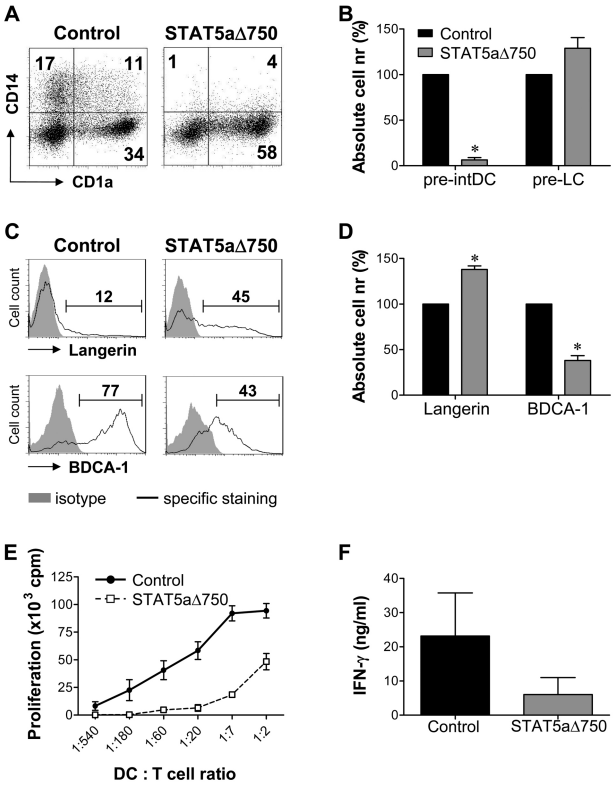

Figure 2. STAT5 activity is required for intDC differentiation, but redundant for LC commitment

CD34⁺ HPC were retrovirally transduced with STAT5aΔ750 or a control vector. ***A)*** *Cells were harvested at day 7 and analyzed for the expression of CD1a and CD14. Representative FACS plots of eGFP⁺ cells are shown (n=7).* ***B)*** *Numbers of eGFP⁺ cells two days after transduction (day 4) were calculated from the trypan blue negative cell count and the percentage of eGFP⁺ cells as determined by FACS. At day 7, the cells were counted and analyzed for the expression of eGFP, CD14 and CD1a. The absolute numbers of eGFP⁺CD14⁺CD1a⁻ pre-intDC and eGFP⁺CD14⁻CD1a⁺ pre-LC were calculated and normalized for the difference in transduction efficiency using the day 4 eGFP⁺ cell count. Shown are mean±SEM pre-intDC and pre-LC absolute cell numbers standardized to control (n=3).* ***CD)*** *eGFP⁺ cells were isolated by FACSorting at day 7 and further cultured under control conditions. Day 10 cells were counted with trypan blue exclusion and analyzed for the expression of Langerin and BDCA-1 by flow cytometry. Numbers of Langerin⁺ and BDCA-1⁺ cells were calculated and standardized to control. Representative FACS plots (C) and mean±SEM absolute cell numbers (D) are shown (n=3).* ***EF)*** *Day 13 cells were stimulated with LPS. After 18 hours, cells were harvested, thoroughly washed and used in allogeneic MLR. T cell proliferation was quantified by incubating the cells with [methyl-³H]thymidine during the last 18 hours of 6-day cultures (E). ELISA determined IFN-γ concentrations in supernatants harvested at day 5 (DC:T cell ratio 1:2) (F). Data represent mean±SD of triplicate cultures of a representative experiment (n=2). * p<0.05, paired Student t-test*

mutant STAT5a1*6. Both introduction of STAT5a-wt and STAT5a1*6 increased STAT5 phosphorylation, but STAT5a1*6 increased STAT5 phosphorylation to a larger extent (Figure 1C). Compared to control cultures, a 44±9% and 51±12% reduction in expansion was observed for STAT5a-wt- and STAT5a1*6-transduced cultures, respectively. More importantly however, myeloid DC development was dramatically reduced in these cultures. Almost no pre-intDC could be observed (Figure 3A,B). Although pre-LC were less affected, the development of these cells was also significantly reduced by STAT5a-wt (Figure 3A,B). STAT5a1*6 further reduced pre-LC development (Figure 3A,B), demonstrating an inverse correlation between the increase in STAT5 activity and pre-LC differentiation capacity. In accordance with the loss of pre-DC, no differentiated DC were present at day 13, as demonstrated by the absence of BDCA-1 and Langerin expression (Figure 3C,D). In addition, the cells generated hardly expressed costimulatory molecules and induced only low T cell proliferation (data not shown). STAT5a-wt- and STAT5a1*6-cultures contained a heterogeneous cell population (Figure 3E). Analysis of lineage-specific surface markers revealed expression of CD71, an early maker of erythrocyte development, but not GPA, which is expressed by fully differentiated erythrocytes (Figure S2). Although STAT5a-wt cultures contained cells whose morphology resembled that of granulocytes (Figure 3E), CD15 expression could not be detected (Figure S2). The leukocyte marker CD45 was expressed by all cells (Figure S2). Whereas control cultures demonstrated almost no proliferation between

day 10 and 13, a 3-fold expansion was observed in the cultures ectopically expressing STAT5 (data not shown). Together, these data demonstrate that increased STAT5 expression abolishes the myeloid DC differentiation capacity of CD34⁺ HPC.

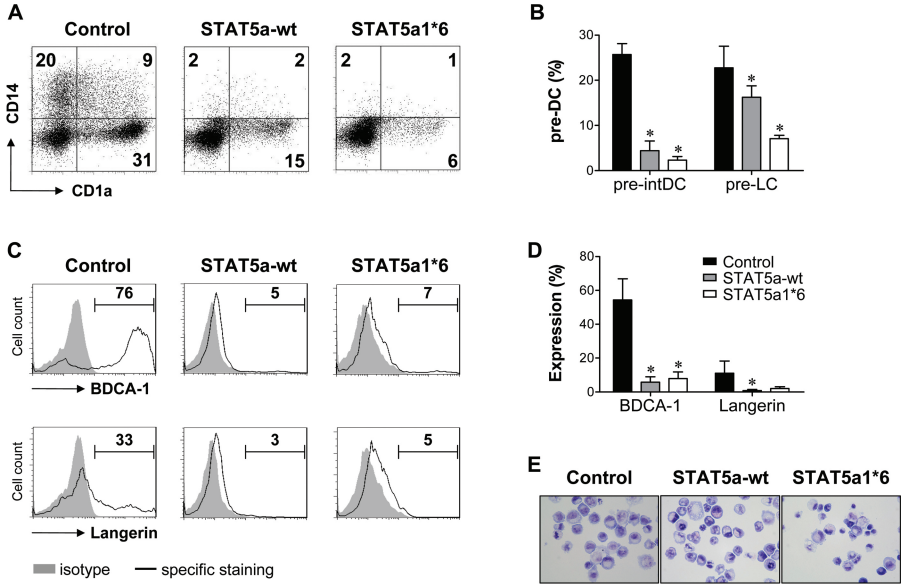

Figure 3. Increased STAT5 activity in CD34⁺ HPC inhibits myeloid DC commitment
*CD34⁺ HPC were retrovirally transduced with STAT5a-wt, STAT5a1*6 or a control vector.* ***AB)*** *Cells were harvested at day 7 and analyzed for the expression of CD1a and CD14 by flow cytometry. The percentages CD14⁺CD1a⁻ pre-intDC and CD14⁻CD1a⁺ pre-LC within the eGFP⁺ population were determined. Representative FACS plots of eGFP⁺ cells (A) and mean±SEM percentage pre-DC within the eGFP⁺ population (B) are shown (n=6).* ***CD)*** *Day 13 cells were analyzed for the expression of BDCA-1 and Langerin by flow cytometry. Representative FACS plots of eGFP⁺ cells (C) and mean±SEM percentage BDCA-1⁺ and Langerin⁺ cells within the eGFP⁺ population (D) are shown (n=3).* ***E)*** *eGFP⁺ cells were isolated by FACSorting at day 7. At day 13, cells were harvested. Cytospins were prepared, stained with May-Grünwald Giemsa solution and analyzed by light microscopy. The results are representative of 3 experiments. Original magnification x400. * p<0.05, paired Student t-test*

STAT5 promotes intDC differentiation from pre-intDC

Since GM-CSF is an important inducer of myeloid DC differentiation as well as STAT5 activation (18, 21), the negative effects of increased STAT5 activity on DC development were surprising. We therefore retrovirally transduced HPC with 4-hydroxytamoxifen (4-OHT)-inducible STAT5a1*6-ER, which allows specific modulation of STAT5 activity levels at different time points during differentiation. Similar to the effects of STAT5a1*6 expression (Figure 3), immediately increasing STAT5 activity by addition of 4-OHT to STAT5a1*6-ER-transduced HPC from the moment of transduction blocked pre-DC differentiation (data not shown). To determine the effect of increased STAT5 activity in purified committed pre-intDC, STAT5a1*6-ER-transduced cells were cultured without 4-OHT until day 7. CD14⁺CD1a⁻ pre-intDC were then isolated and differentiated towards intDC in the presence or absence of 4-OHT. As previously reported (24), terminal differentiation of pre-intDC to intDC involves the acquisition of CD1a (Figure 4A). Conditional STAT5 activation by addition of 4-OHT resulted in increased CD1a expression (Figure 4A,B), suggesting that STAT5 increased differentiation. After one day the ratio of CD1a⁺ and CD1a⁻ cells was similar for control and 4-OHT cultures, but two and three days incubation with 4-OHT resulted in a relative increase in CD1a⁺ cells compared to control cultures (Figure 4B). No difference in viability was found (Figure 4C), resulting in similar cell yields in control and 4-OHT cultures (Figure S3A). The similar yields and increased percentage of CD1a⁺ cells resulted in a variable but consistent

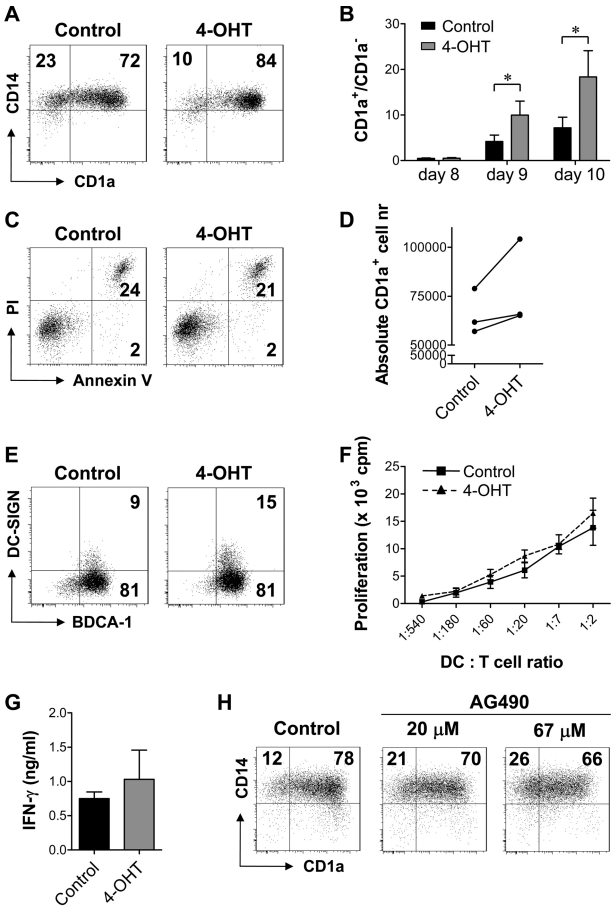

Figure 4. STAT5 promotes intDC differentiation from purified pre-intDC

*CD34⁺ HPC were retrovirally transduced with 4-OHT-inducible STAT5a1*6-ER. At day 7, transduced pre-intDC (iNGFR⁺ CD14⁺CD1a⁻) were isolated by FACSort. Cells were further cultured in the presence of either 4-OHT or its solvent EtOH.* ***AB)*** *Cells were harvested at days 8, 9 and 10 and analyzed for the expression of CD14 and CD1a. The percentages CD1a⁻ and CD1a⁺ cells were determined and the ratio was calculated. Representative FACS plots of day 9 cells (A) and mean±SEM CD1a⁺/CD1a⁻ ratios (B) of at least 4 independent experiments with different donors are shown.* ***C)*** *Day 8 cells were analyzed for AnnexinV/PI staining. Representative FACS plots are shown (n=4).* ***D)*** *Day 9 cells were harvested, counted with trypan blue exclusion and analyzed for the expression of CD1a by flow cytometry. Absolute numbers of CD1a⁺ cells were calculated. Three independent experiments with different donors are shown.* ***E)*** *Representative FACS plots of at least 3 independent experiments showing DC-SIGN and BDCA-1 expression at day 10.* ***FG)*** *Day 10 cells were stimulated with LPS. At day 12, cells were harvested, thoroughly washed and used in allogeneic MLR. T cell proliferation was quantified by incubating the cells with [methyl-³H]thymidine during the last 18 hours of 6-day cultures (F). ELISA determined IFN-γ concentrations in supernatants harvested at day 5 (DC:T cell ratio 1:2) (G). Data represent mean±SD of triplicate cultures of a representative experiment (n=3).* ***H)*** *CD34⁺ HPC were cultured under control conditions until day 7. Then, CD14⁺CD1a⁻ pre-intDC were isolated by FACSort and further cultured in the presence or absence of JAK2 inhibitor AG490. At day 9, cells were harvested and analyzed for the expression of CD1a and CD14 by flow cytometry. Representative FACS plots of 3 independent experiments with different donors are shown. * p<0.05, paired Student t-test*

increase of absolute CD1a⁺ cell numbers in three independent experiments (Figure 4D). These data support the view that conditional STAT5 activation improved differentiation rather than induced apoptosis of the less differentiated CD1a⁻ cells. Enhanced differentiation was further demonstrated by the augmented DC-SIGN expression, whereas most cells expressed BDCA-1 irrespective of 4-OHT addition (Figure 4E). None of these effects were observed when 4-OHT was added to control cells, either untransduced or transduced with an empty control vector (Figure S4A-D and data not shown), confirming that the observed effects resulted specifically from increased STAT5 activation. Furthermore, DC generated in the presence of increased STAT5 activation were functional, as demonstrated by the normal LPS-induced CD86 and HLA-DR expression (Figure S3B). T cell stimulatory capacity was also unaffected and 4-OHT-treated DC even modestly increased IFN-γ secretion by allogeneic T cells (Figure 4F,G).

To confirm the importance of STAT5 in terminal intDC differentiation and to investigate the function of endogenous STAT5 activity, JAK2-STAT5 signaling was inhibited in pre-intDC by treatment with the JAK2 inhibitor AG490. JAK2 inhibition impaired differentiation, as demonstrated by a modest but reproducible reduction in CD1a expression (67 μM: 16±2%; $p<0.05$) (Figure 4H). Taken together, these data show that although increased STAT5

activation negatively affects DC development from CD34⁺ HPC, it has a beneficial effect on the differentiation of cells that have already committed to the DC lineage, leading to improved differentiation of pre-intDC to intDC.

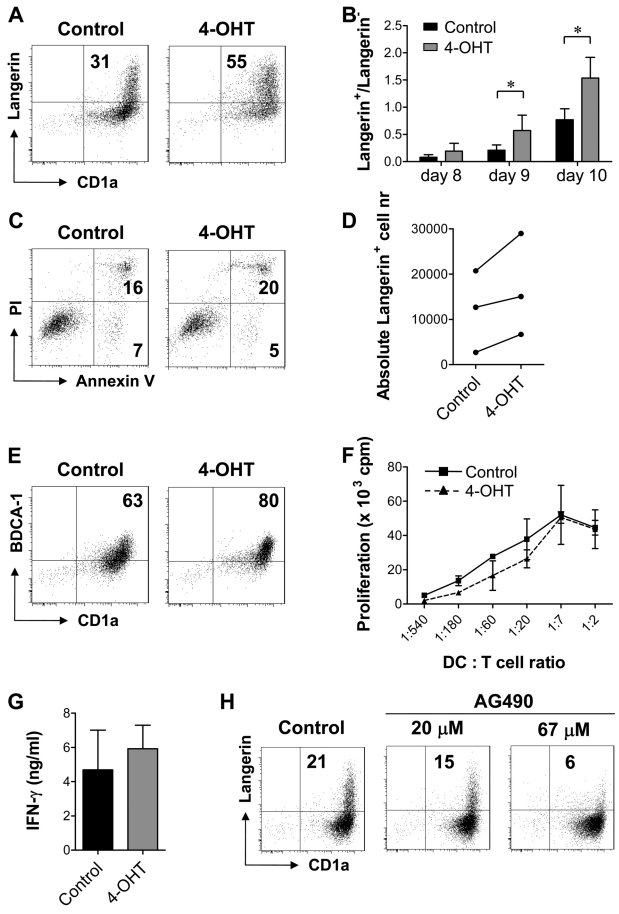

Figure 5. STAT5 promotes LC differentiation from purified pre-LC

*CD34⁺ HPC were retrovirally transduced with 4-OHT-inducible STAT5a1*6-ER. At day 7, transduced pre-LC (iNGFR⁺CD14⁻ CD1a⁺ cells) were isolated by FACSort. Cells were further cultured with 4-OHT or EtOH.* ***AB)*** *Cells were harvested at days 8, 9 and 10 and analyzed for the expression of CD1a and Langerin by flow cytometry. The percentages Langerin⁻ and Langerin⁺ cells were determined and the ratio was calculated. Representative FACS plots of day 9 cells (A) and mean±SEM Langerin⁺/Langerin⁻ ratios (B) of at least 3 independent experiments with different donors are shown.* ***C)*** *Day 8 cells were analyzed for AnnexinV/PI staining. Representative FACS plots are shown (n=4).* ***D)*** *Day 9 cells were harvested, counted with trypan blue exclusion and analyzed for the expression of Langerin by flow cytometry. Absolute numbers of Langerin⁺ cells were calculated. Three independent experiments with different donors are shown.* ***E)*** *Representative FACS plots showing CD1a and BDCA-1 expression at day 10 (n=3).* ***FG)*** *Day 10 cells were stimulated with LPS. At day 12, cells were harvested, thoroughly washed and used in allogeneic MLR. T cell proliferation was quantified by incubating the cells with [methyl-³H]thymidine during the last 18 hours of 6-day cultures (F). ELISA determined IFN-γ concentrations in supernatants harvested at day 5 (DC:T cell ratio 1:2) (G). Data represent mean±SD of triplicate cultures of a representative experiment (n=2).* ***H)*** *CD34⁺ HPC were cultured under control conditions until day 7. Then, CD14⁻CD1a⁺ pre-LC were isolated by FACSort and further cultured in the presence or absence of JAK2 inhibitor AG490. At day 9, cells were harvested and analyzed for the expression of CD1a and Langerin by flow cytometry. Representative FACS plots of 3 independent experiments with different donors are shown. * p<0.05, paired Student t-test*

STAT5 promotes LC differentiation from pre-LC

We next investigated the consequences of increasing STAT5 activity in purified pre-LC. Similar to pre-intDC, a time-dependent relative increase in differentiated cells was observed in 4-OHT-treated cultures compared to control, as demonstrated by the increased percentage of Langerin⁺ cells (Figure 5A,B). Although already evident after one day, this became increasingly clear at later time points (Figure 5B). Viability and cell yields were relatively unaffected one and two days after 4-OHT addition (Figure 5C and Figure S3C), resulting in increased Langerin⁺ cell yields at day 9 (Figure 5D). We have previously shown that LC generated in this culture system are more susceptible to apoptotic death after terminal differentiation than intDC (24). In accordance, the enhanced LC differentiation was accompanied by increased apoptosis and reduced cell yields at day 10 (Figure S3C,D). In addition to Langerin, BDCA-1 expression was also increased in 4-OHT-treated cultures (Figure 5E). In control- or untransduced cultures, 4-OHT did not affect LC development (Figure S4E-I and data not shown). Similar to intDC, 4-OHT-treated LC exhibited normal

LPS-induced maturation (Figure S3E) and T cell stimulatory capacity (Figure 5F,G). The importance of STAT5 in terminal LC differentiation was further confirmed by the reduced Langerin expression in JAK2-inhibited cultures (Figure 5H). Taken together, these data demonstrate that STAT5 activation promotes terminal differentiation of pre-LC.

STAT5 modulates DC lineage commitment by regulation of PU.1 expression levels

Although STAT5 activity promoted terminal LC differentiation from pre-LC (Figure 5), STAT5 was redundant for and even impeded LC commitment of HPC, as demonstrated by the LC skewing induced by introduction of dominant-negative STAT5 (Figure 2). The latter was remarkably similar to the reported LC skewing induced by TGF-β (27), a finding that was confirmed by the increased Langerin, E-cadherin and cutaneous leukocyte antigen expression we found upon TGF-β addition to our cultures (Figure 6A and data not shown). To investigate this further, we analyzed the effects of TGF-β addition on STAT5 activity. As determined by both FACS and western blot, TGF-β reduced STAT5 phosphorylation in CD34-derived myeloid DC cultures at day 0 and day 10 (Figure 6B,C). This was associated with a significant increase in PU.1 (Figure 6C), a protein whose expression is known to be upregulated by TGF-β (28). In addition, PU.1 expression in STAT5aΔ750 cultures was somewhat increased compared to control, whereas PU.1 levels were dramatically reduced in cells with increased STAT5 activity (Figure 6D). Furthermore, the expression of CD11b, which is transcriptionally regulated by PU.1 (29-30), was also inversely correlated with STAT5 activity (Figure 6E). Since PU.1 is a critical transcription factor for DC development (31), these data suggest that the regulation of CD34-derived myeloid DC commitment by STAT5 is at least partly mediated by regulation of PU.1 expression.

Discussion

DC, the most important antigen presenting cells, are relatively short-lived and therefore need to be continuously replenished. However, our knowledge on how this is achieved remains limited. Here, we show that STAT5 is active at all stages of differentiation during human CD34-derived intDC and LC development, but that higher activation levels are present in further differentiated cells. Tight regulation of STAT5 transcription factor activity is required for optimal development of these myeloid DC. Depending on the DC developmental stage, manipulation of STAT5 activity levels results in (a) skewing towards a specific myeloid DC subset, (b) a complete blockade in myeloid DC development, or (c) enhanced myeloid DC differentiation.

While intDC development required STAT5 activity, this transcription factor was redundant for and even impeded the differentiation of $CD34^+$ HPC to pre-LC. The relatively low endogenous STAT5 activity during development of pre-DC from HPC, together with our finding that ectopic activation of STAT5 in early progenitors abolished commitment to the DC lineage, indicates that STAT5 activity needs to be carefully regulated. It was speculated very recently that STAT5 activation promotes development of mouse conventional DC (22, 32), a DC lineage related to the human myeloid DC subsets (6). However, our data not only show myeloid subset-specific requirements for STAT5 activity, but also clearly demonstrate that this process is not regulated in a simple on-off binary manner. Instead, careful regulation of the level of STAT5 activity appears crucial.

The skewing towards the LC lineage upon inhibition of STAT5 activity showed similarities with TGF-β-induced LC commitment as previously reported (27-28). In line with previous studies demonstrating TGF-β-induced inhibition of STAT5 transcription factor activity (33-35), we observed reduced STAT5 phosphorylation upon addition of TGF-β. Furthermore, both

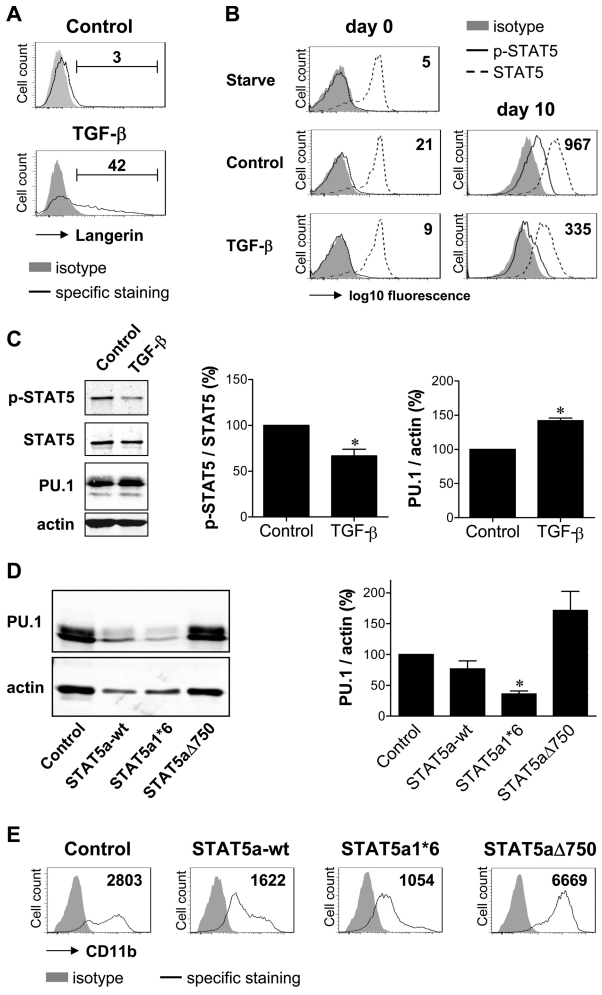

Figure 6. STAT5 modulates DC lineage commitment by regulation of PU.1 expression levels

A) *CD34⁺ HPC were cultured to myeloid DC in the presence or absence of TGF-β. Cells were harvested at day 10 and analyzed for the expression of Langerin by flow cytometry. Representative FACS plots of 3 independent experiments with different donors are shown.* ***B)*** *Cells cultured without any added cytokines, under control conditions or under control conditions in the presence of TGF-β were harvested at day 0, 6 hours after the start of culture. Day 10 cells had been cultured in the presence or absence of TGF-β from day 0. Cells were fixed and intracellular FACS staining for phoshorylated and total STAT5 was performed. Specific p-STAT5 staining, calculated by subtracting the mean fluorescence intensity of the isotype control from the mean fluorescence intensity measured for p-STAT5, is indicated by the numbers in the FACS plots. Data are representative of 4 (day 0) or 10 (day 10) independent experiments with different donors.* ***C)*** *Cells cultured in the presence or absence of TGF-β were harvested at day 10. Total cell lysates were prepared and the expression of phosphorylated STAT5, total STAT5, PU.1 and actin was determined by western blot. Ratios of phosphorylated and total STAT5 were calculated and standardized to control. PU.1 expression was normalized to actin and standardized to control. A representative experiment, mean±SEM p-STAT5/STAT5 ratios and mean±SEM PU.1 expression of 3 independent experiments with different donors are shown.* ***D)*** *CD34⁺ HPC were retrovirally transduced with STAT5a-wt, STAT5a1*6, STAT5aΔ750 or a control construct. Transduced cells were FACSorted at day 7. Total cell lysates were prepared and PU.1 and actin expression were determined. PU.1 expression was normalized to actin and standardized to control. A representative experiment and mean±SEM PU.1 expression of 3 independent experiments with different donors are shown.* ***E)*** *CD34⁺ HPC were transduced as described in (D). At day 7, CD11b expression of eGFP⁺ cells was determined by flow cytometry. Representative FACS plots are shown (n=3). Specific staining, calculated by subtracting the mean fluorescence intensity of the isotype control from the mean fluorescence intensity measured for CD11b, is indicated by the numbers in the FACS plots. * p<0.05, paired Student t-test*

exposure to TGF-β and inhibition of STAT5 transcription factor activity were associated with an increased expression of PU.1, a critical transcription factor for DC development (28, 31, 36-37). These data suggest that TGF-β-induced LC skewing may be mediated by inhibition of STAT5 activity, resulting in elevated PU.1 expression levels favorable for LC commitment (28, 38) (Figure 7). Since PU.1 has an instructive role in cytokine-driven DC development (28, 31), but cannot replace the cytokines inducing DC differentiation (28), PU.1 can only favor DC development within the proper micro-environment. Therefore, the seeming contradiction between the increased PU.1 expression but inhibited intDC development upon STAT5 inhibition could be explained by other transcriptional targets of STAT5 involved in intDC development (Figure 7).

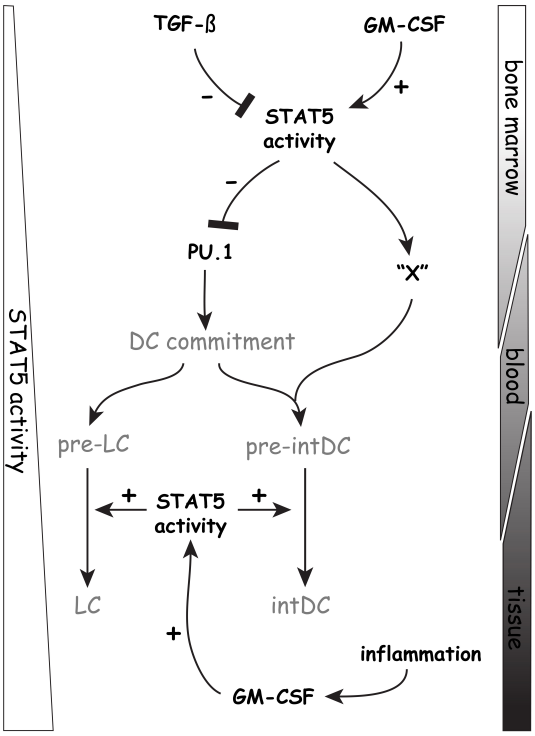

Figure 7. STAT5 regulates human intDC and LC development

STAT5 activation is determined by the combined action of various cytokines. The role of STAT5 in the regulation of human myeloid DC development is subset-specific. While LC commitment is promoted by TGF-β-induced inhibition of STAT5 activity and subsequent upregulation of PU.1, pre-intDC differentiation requires STAT5 transcription factor activity. However, high STAT5 activity levels impair DC commitment by inhibition of the critical DC transcription factor PU.1, indicating that careful regulation of STAT5 activity levels is essential. In addition, the timing of STAT5 activation is crucial. In contrast to the inhibition of DC commitment by high STAT5 activation levels during HPC to pre-DC differentiation, in committed pre-DC, terminal DC differentiation is promoted by activation of STAT5. The altered need for STAT5 activity at distinct developmental stages fits with the idea that systemic levels of GM-CSF are normally low, while pre-DC meet high concentrations of GM-CSF when they are attracted to sites of inflammation that are in urgent need for functional DC.

The inverse correlation between STAT5 activity and PU.1 expression could also be responsible for the block in myeloid DC development upon ectopic STAT5 activation in HPC. PU.1 is indispensable for both myelopoiesis (39) and DC development (31, 36, 40). Thus, the loss of PU.1 upon increased STAT5 activity could account for the reported block in myelopoiesis (12, 14) and the abolished DC development shown here. Although we cannot fully exclude that PU.1 loss is the consequence rather than the cause of reduced DC development, STAT5a1*6-ER transduced HPC stimulated for 24 hours with 4-OHT demonstrated 40% reduction in PU.1 mRNA levels (A.T.J. Wierenga, unpublished data), supporting the view that PU.1 expression is regulated by STAT5. Moreover, a recent study showed that increased STAT5 activity inhibits myelopoiesis and promotes erythrocyte differentiation through GATA-1, a functional antagonist of PU.1 (41). Although no mature erythrocytes could be observed in the present study, the induction of CD71 expression by increased STAT5 activity suggests partial differentiation towards the erythrocyte lineage. Erythrocyte differentiation has been shown to benefit from ectopic STAT5 expression (16), but their full differentiation probably requires more than STAT5 activation alone.

While STAT5 activation should be low in $CD34^+$ HPC to allow DC commitment, increased STAT5 phosphorylation was observed during pre-DC to DC development and ectopic activation of STAT5 in pre-DC improved their terminal differentiation. Although seemingly contradictory, altered roles of STAT5 at distinct developmental stages appear relevant in vivo. Serum concentrations of GM-CSF and other STAT5-activating cytokines are normally low (42), inducing low levels of STAT5 activation optimal for the commitment of early hematopoietic progenitors to DC lineages. However, pre-DC not only continuously replenish DC during steady state, but are also attracted to sites of infection or inflammation to locally increase the DC pool (43). The high GM-CSF concentrations at these sites will result in increased STAT5 activation in the pre-DC, aiding in their prompt differentiation to

fully functional DC (Figure 7). Unfortunately, currently available in vivo models to investigate human DC development do not provide the opportunity of differential manipulation during distinct phases of DC differentiation. It will therefore remain a major challenge to confirm the important role of STAT5 as shown here in an in vivo model that allows careful regulation of STAT5 activity at different stages of DC development. In addition, replication of the current data in an in vitro system that includes Flt3 ligand to better mimic the cytokine situation in the steady state would be interesting, as the combination of GM-CSF, SCF and TNF-α used in the present study may contribute to a more pro-inflammatory environment than generally present in vivo.

Enhanced differentiation induced by increased STAT5 activation in pre-DC did not reduce the functionality of the resulting DC. Furthermore, these intDC even modestly increased IFN-γ secretion by allogeneic T cells. This could indicate a direct role for STAT5 in DC function, which would be in accordance with previous studies suggesting regulation of DC maturation or function by JAK2-STAT5 signaling (44-46). Alternatively, the increased proportion of BDCA-3$^+$ DC in these cultures (L. van de Laar, unpublished data) could also contribute to Th1 skewing, as the recently characterized BDCA-3$^+$ DC were shown to promote Th1 differentiation (47).

In conclusion, we show a critical function for STAT5 in controlling development of human intDC and LC. STAT5 is redundant for LC commitment, but required to induce pre-intDC differentiation of HPC. However, STAT5 activation levels should be low to allow commitment to either DC lineage. In contrast, high STAT5 activity promotes terminal differentiation of already committed pre-DC. Thus, specific requirements for the level of STAT5 activation exist at distinct stages of intDC and LC differentiation. Regulation of the STAT5 activation status may therefore be one of the mechanisms by which cytokines determine the fate of possible DC precursors at various locations under different pathophysiological conditions. Fundamental insight in the intracellular signal transduction pathways and transcription factors involved in human DC development will improve the understanding of how continuous DC replenishment from their hematopoietic progenitors is regulated and homeostasis of these important immune cells is maintained.

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Data supplement

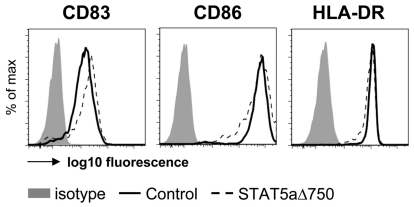

Figure S1. Similar expression of costimulatory molecules by DC in control and STAT5aΔ750 cultures
CD34⁺ HPC were transduced, cultured and FACSorted as for Figure 2E,F. LPS was added from day 13. At day 15, cells were analyzed for the expression of CD1a, BDCA-1, CD83, CD86 and HLA-DR. Representative FACS plots show CD83, CD86 and HLA-DR expression by CD1a⁺BDCA1⁺-gated DC (n=3).

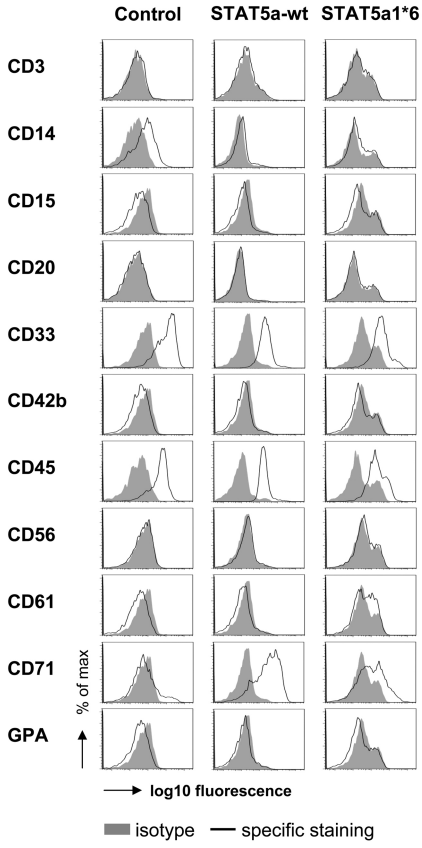

Figure S2. Expression of lineage markers
CD34⁺ HPC were transduced and FACSorted as for Figure 3E. The expression of CD3, CD14, CD15, CD20, CD33, CD42b, CD45, CD56, CD61, CD71 and GPA was analyzed at day 13. Representative FACS plots are shown (n=2).

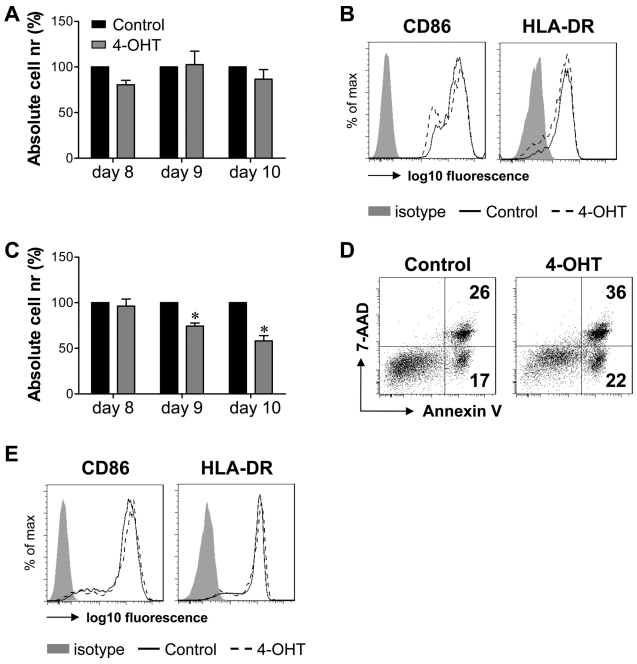

Figure S3. Effects of conditional STAT5 activation on yields, survival and costimulatory molecule expression

CD34⁺ HPC were transduced, FACSorted and cultured as described in Figure 4 (AB) and 5 (C-E). ***AC)*** *At days 8, 9 and 10, cells were harvested and counted with trypan blue exclusion. Cell yields were standardized to the control cultures. Data represent mean±SEM standardized yields of at least 3 independent experiments with different donors.* ***BE)*** *LPS was added from day 10. Representative FACS plots show CD86 and HLA-DR expression at day 12 (n=3).* ***D)*** *Day 10 cells were analyzed for AnnexinV/7-AAD staining. Representative FACS plots are shown (n=3). * p<0.05, paired Student t-test*

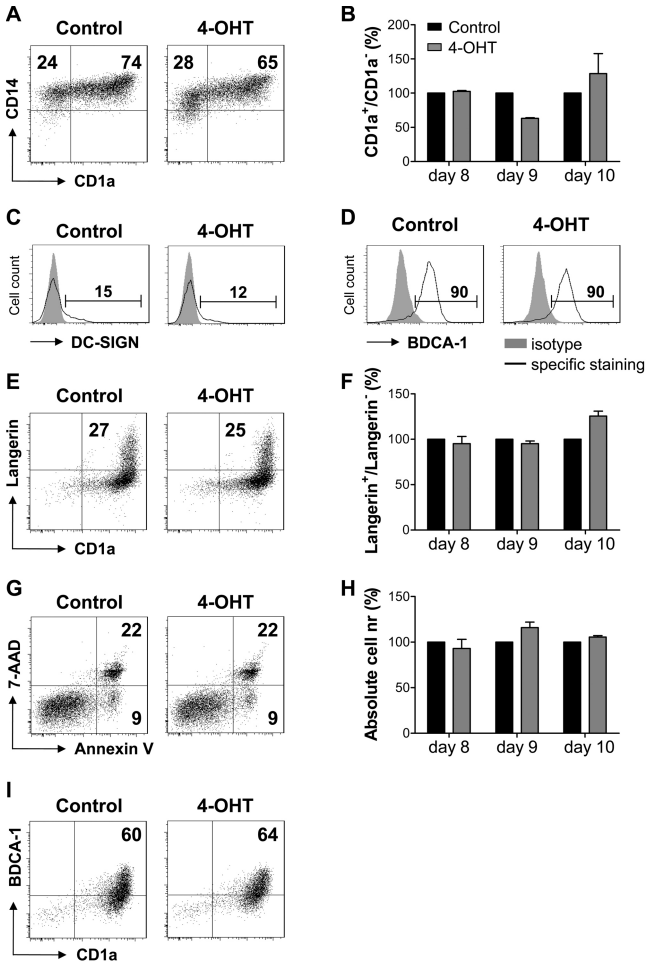

Figure S4. Treatment of control pre-DC with 4-OHT

Control CD14$^+$CD1a$^-$ pre-intDC (A-D) or CD14$^-$CD1a$^+$ pre-LC (E-I) were FACSorted at day 7 and further cultured with 4-OHT or EtOH. Data are representative of at least 2 independent experiments with different donors. ***AB)*** *Cells were analyzed for CD1a and CD14 expression at days 8, 9 and 10. The percentages CD1a$^-$ and CD1a$^+$ cells were determined, and the ratios were calculated and standardized to the control cultures. Representative FACS plots of day 9 cells (A) and mean±SEM CD1a$^+$/CD1a$^-$ ratios (B) are shown.* ***CD)*** *Representative FACS plots showing DC-SIGN and BDCA-1 expression at day 10.* ***EF)*** *Cells were analyzed for CD1a and Langerin expression at days 8, 9 and 10. The percentages of Langerin$^-$ and Langerin$^+$ cells were determined, and the ratios were calculated and standardized to the control cultures. Representative FACS plots of day 9 cells (E) and mean±SEM Langerin$^+$/Langerin$^-$ ratios (F) are shown.* ***G)*** *Cells were analyzed for AnnexinV/7-AAD staining at day 10. Representative FACS plots are shown.* ***H)*** *Cells were harvested at days 8, 9 and 10 and counted with trypan blue exclusion. Data represent mean±SEM cell yields, standardized to control.* ***I)*** *Representative FACS plots showing CD1a and BDCA-1 expression at day 10.*

6

ENHANCED PI3K-PKB ACTIVITY AUGMENTS HUMAN PLASMACYTOID DENDRITIC CELL DEVELOPMENT AND FUNCTION

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Abstract

Due to their key role in the regulation of immunity, plasmacytoid dendritic cells (pDC) are considered potential tools or targets for immunotherapy. However, the current knowledge concerning methodologies to manipulate their development or function remains limited. Here, we investigated the role of the PI3K-PKB-mTOR axis in human pDC development, survival and function. In vitro pDC generation from human cord blood-derived $CD34^+$ hematopoietic progenitor cells was strongly reduced by pharmacological inhibition of PI3K, PKB or mTOR activity. Apoptosis likely contributed to the pDC loss, since peripheral blood pDC required PI3K-PKB-mTOR signaling to survive. Importantly, the PI3K-PKB-mTOR pathway was not only essential, but increasing its activity also promoted pDC development and function. Enhanced PKB activation during in vitro as well as in vivo CD34-derived pDC development resulted in increased pDC numbers and augmented MHC class II and costimulatory molecule expression. In addition, production of IFN-α and TNF-α was increased in both CD34-derived and peripheral blood-derived pDC. Finally, TLR-induced PI3K-PKB-mTOR-S6K activation was reduced in the functionally impaired pDC isolated from blood samples of chronic hepatitis B virus patients. In conclusion, intact PI3K-PKB-mTOR signaling is required for the development and survival of human pDC, and increased activation of this pathway promotes pDC development and functionality. Manipulation of this pathway or its downstream targets could be used to improve the generation and function of pDC in order to augment immunity.

Introduction

Plasmacytoid dendritic cells (pDC) represent a specialized DC subset that is recognized for its unique ability to rapidly produce large amounts of type 1 interferons (IFN-αβ) upon the interaction with viruses or nucleic acids of self or non-self origin (1). Besides direct anti-viral activity through the production of IFN-α, pDC have been implicated in playing a broader role in immunity. pDC activation results in the production of additional pro-inflammatory cytokines such as TNF-α and IL-6, and stimulates the expression of MHC class I, MHC class II and costimulatory molecules. These combined functions enable pDC to activate T helper cells, cytotoxic T lymphocytes, NK cells and plasma cell differentiation. Moreover, pDC-derived cytokines can act as adjuvants for other DC subsets such as myeloid or conventional DC. In contrast, tolerogenic functions of pDC, including the generation of regulatory T cells, have also been described. Similar to other DC subsets, pDC thus show the dual capability of being able to initiate as well as regulate immunity (1-3).

In accordance with their broad role in the regulation of immunity, the possibilities to use pDC as tools or targets for immunotherapy are being increasingly explored. Activation of dysfunctional tumor-resident pDC may be achieved by administration of TLR agonists (4-5), and methods to induce anti-tumor responses by either targeting antigen to endogenous pDC or injection of antigen-loaded pDC are currently being investigated (6-8). In addition, the reduced pDC numbers and functionality observed in patients with persistent viral infections such as human immunodeficiency virus, hepatitis B virus (HBV) and hepatitis C virus has prompted research aiming to manipulate patient pDC to promote anti-viral immunity (9-11). The development of strategies to manipulate pDC immunity would greatly benefit from the identification of intracellular signal transduction pathways or transcription factors that regulate differentiation, survival and function of pDC. However, our current knowledge concerning methodologies to manipulate molecular mechanisms involved in pDC development and functionality remains limited.

In mice, mammalian target of rapamycin (mTOR) has recently been associated with both TLR9-induced IFN-α production and pDC development (12-13). mTOR activation is achieved through phosphatidylinositol 3-kinase (PI3K) (14-15). PI3K generates 3-phosphorylated inositol lipids causing activation of downstream signaling resulting in the activation of protein kinase B (PKB; also called c-AKT), which regulates, among others, mTOR complex 1 (mTORC1), glycogen synthase kinase-3β and Forkhead box O transcription factor activity. Phosphatase and tensin homolog (PTEN) acts as a cell-intrinsic negative regulator of PI3K-dependent signaling through dephosphorylation of 3-phosphorylated inositol lipids (15).

The PI3K-PKB-mTOR signaling pathway has been shown to regulate the differentiation and function of human myeloid DC (16-21), but the role of this pathway in human pDC biology remains undefined. In the present study, we show that this signaling module regulates the development, survival and function of human pDC. Pharmacological inhibition of PI3K, PKB or mTOR resulted in reduced pDC development and survival, and impaired PI3K-PKB-mTOR-S6 Kinase (S6K) activation was found in dysfunctional pDC present in the blood of patients chronically infected with HBV. In vitro and in vivo pDC development from human cord blood-derived $CD34^+$ hematopoietic precursors was significantly increased following ectopic activation of PKB. Moreover, increased activation of this pathway augmented the expression of MHC class II and costimulatory molecules, and enhanced stimulation-induced production of IFN-α and TNF-α. These findings not only demonstrate the crucial role of the PI3K-PKB-mTOR pathway in human pDC biology, but may also provide new approaches to manipulate pDC development, survival and function in order to regulate immunity.

Materials and Methods

Reagents

Where indicated the pharmacological inhibitors LY294002 (LY; 10 μM unless indicated otherwise; Biomol, Plymouth Meeting, PA), PKB inhibitor VIII (VIII; 3 μM unless indicated otherwise; Calbiochem, La Jolla, CA), Rapamycin (Rapa; 1 μM unless indicated otherwise; Biomol) or VO-OHpic trihydrate (VO-OHpic; 10 nM; Sigma-Aldrich, St. Louis, MO) were added to the cultures. HSV-1 (HSV; MOI 10) was provided by G.M.G.M Verjans (Dept of Virology, Erasmus MC-University Medical Center Rotterdam, The Netherlands). Synthetic TLR ligands included CpG-2336 (CpG; class A; 10 μg/ml; Coley Pharma, Düsseldorf, Germany) and Loxoribine (Lox; 0.4 mM; Invivogen, San Diego, CA).

Culture of pDC

Peripheral blood pDC: PBMC were isolated from buffy coats obtained from healthy blood donors using Ficoll (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. pDC were isolated from the PBMC fraction by $CD19^+$ cell depletion followed by positive selection using anti-BDCA-4-PE and anti-PE-coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated pDC were analyzed for purity and viability by staining for BDCA-2 (Miltenyi) and 7-AAD (eBioscience, San Diego, CA), and only used for experiments if over 95% pure and viable. pDC were cultured in RPMI-1640 (Lonza, Breda, The Netherlands) containing 8% heat inactivated fetal bovine serum (FBS; Sigma-Aldrich), penicillin/streptomycin (Invitrogen, Breda, The Netherlands), 2 mM L-glutamine (Lonza), 10 mM HEPES (Lonza) and 20 ng/ml IL-3 (Miltenyi). *CD34-derived pDC:* Umbilical cord blood samples were obtained ex-uterine according to legal guidelines. $CD34^+$ cells were isolated from mononuclear fractions through positive selection using anti-CD34-coated microbeads and MS separation columns (both Miltenyi Biotec GmBH, Bergish Gladbach, Germany) to a purity of 85-98%, used immediately or stored at -135ºC until use. pDC were generated by co-culture of 12.500 OP9-Control cells (22-23) and 25.000 $CD34^+$ cells in MEMα (Invitrogen) containing 20% heat inactivated FBS, penicillin/streptomycin, 5 ng/ml Flt3 ligand (Flt3L; Peprotech, London, UK) and 5 ng/ml IL-7 (Peprotech). Cytokines were refreshed after one week and cells were analyzed after 2 weeks of co-culture unless indicated otherwise.

Retroviral transduction of $CD34^+$ cells

The retroviral DNA constructs LZRS-myrPKB-IRES-eGFP and LZRS-IRES-eGFP, retrovirus production using the retroviral packaging cell line Phoenix-ampho and transduction of $CD34^+$ progenitors with viral supernatant in retronectin-coated wells were described before (19). For in vitro cultures, cells were transduced in RPMI-1640 supplemented with 8% heat inactivated FBS, penicillin/streptomycin, 20 ng/ml Flt3L, 20 ng/ml thrombopoietin (Peprotech) and 10 ng/ml IL-7. $CD34^+$ progenitors used for in vivo mouse experiments were transduced in IMDM medium (Invitrogen) supplemented with 8% heat inactivated FBS, penicillin/streptomycin, 50 μM β-mercaptoethanol (Merck, Darmstadt, Germany), 2 mM L-glutamine, 50 ng/ml Flt3L and 50 ng/ml stem cell factor (Peprotech).

Analysis of cell surface phenotype, intracellular cytokines and apoptosis by flow cytometry

Surface markers: Labeling was performed on ice in PBS containing 1% bovine serum albumin (BSA), 1% heat inactivated human serum and 0.02% NaN_3, using fluorochrome-conjugated antibodies against BDCA-2 (AC144, Miltenyi Biotec), BDCA-4 (AD5-17F6, Miltenyi Biotec), HLA-DR (LN3, eBioscience), CD40 (5C3, eBioscience), CD86 (2331 (FUN-1), BD Biosciences, Breda, The Netherlands) and CD123 (biotin-conjugated; 6H6, eBioscience). Binding of biotin-conjugated antibodies was visualized by a second incubation with fluorochrome-conjugated streptavidin (BD Biosciences). *Intracellular proteins:* The frequencies of IFN-α and TNF-α producing pDC were quantified by incubating cells with 10 μg/ml Brefeldin A (Sigma-Aldrich) during the last 3 hours of 5 hour stimulation cultures. Prior to incubation with antibodies against IFN-α (MMHA-11, PBL InterferonSource, Piscataway, NJ) and TNF-α (MAb11, eBioscience), peripheral blood pDC were fixed (30 minutes) and subsequently permeabilized (15 minutes) by incubation in 2% formaldehyde (Merck) and 0.5% saponine (VWR, Amsterdam, The Netherlands). Labeling was performed on ice in 0.5% saponine. Transduced CD34-derived cultures were first labeled for pDC on ice. To preserve eGFP expression, these cells were then fixed (15 minutes) and subsequently permeabilized (5 minutes) by incubation in fixation medium and permeabilization medium (Fix&Perm, ADG Bio Research, Kaumberg, Austria). Labeling of IFN-α (225.C, Chromaprobe, Maryland Heights, MO) and TNF-α (MAb11, eBiosciences) was performed in permeabilization medium.

Apoptosis: Apoptosis was detected in Annexin buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Cells were incubated with fluorochrome-conjugated Annexin V (BD Biosciences) for 30 minutes on ice and subsequently taken up in 1 µg/ml Propidium Iodide (PI; Sigma-Aldrich). *Assessment:* Assessment was performed using a FACSCanto II (BD Biosciences) and data were analyzed using FlowJo software (www.flowjo.com). Surface markers and intracellular proteins were analyzed on viable cells, gated on FSC/SSC and Aqua (Invitrogen) staining.

Transplantation of β2-microglobulin$^{-/-}$NOD/SCID mice with human CD34$^+$ progenitors
Protocols for mouse experiments were approved by the local animal experimental committee. The β2-microglobulin$^{-/-}$ nonobese diabetic/severe combined immune deficient (NOD/SCID) mice were bred and maintained under sterile conditions in microisolator cages and provided with autoclaved food and acidified water containing 111 mg/L ciprofloxacin (Ciproxin). 8- to 10-week-old mice, sublethally irradiated with 250 cGy x-rays, received transplants via tail vein injections with approximately 0.5 x 10^6 unsorted retrovirally transduced human cord blood-derived CD34$^+$ hematopoietic progenitors along with 10^6 irradiated (1500 cGy) CD34$^+$-depleted human cord blood-derived accessory cells. Six weeks after transplantation, the mice were sacrificed and both tibiae and femora were flushed. Bone marrow cells were analyzed for the presence of human pDC using flow cytometry.

Analysis of S6 phosphorylation in blood samples of HBV patients and healthy controls
Peripheral heparinized blood samples were obtained from 6 patients with chronic hepatitis B. All patients were negative for antibodies against hepatitis C, hepatitis D and human immunodeficiency virus, and did not receive treatment at time of blood donations. A matched control group comprised 3 healthy subjects. The study was approved by the local ethics committee and all subjects gave informed consent before blood donation. PBMC were isolated using Ficoll density gradient centrifugation and cryopreserved until use. PBMC from age and gender matched healthy subjects, HBeAg-positive and HBeAg-negative HBV infected patients were simultaneously processed by incubating 10^6 PBMC in 250 µl X-vivo medium (Lonza), supplemented with penicillin/streptomycin, 2 mM L-glutamine and 10 mM HEPES at 37°C for 5 hours. CpG or Lox were added 15 minutes, 1 hour, 2 hours or 4 hours before the end of culture. Fluorochrome-conjugated antibodies against CD11c and BDCA-4 were added during the last 5 minutes. After 5 hours, cultures were fixed (10 minutes at 37°C) and subsequently permeabilized (30 minutes on ice) by incubation in lyse/fix buffer (BD Biosciences) and PERM buffer III (BD Biosciences). Cells were then labeled with fluorochrome-conjugated antibodies against S235/S236-phosphorylated S6 (p-S6; N7-548, BD Biosciences) for 30 minutes on ice.

Results

CD34-derived pDC development requires PI3K-PKB-mTOR signaling
The importance of the PI3K-PKB-mTOR signaling module in pDC development was first investigated in vitro. Generation of CD123$^+$BDCA-2$^+$ pDC from CD34$^+$ hematopoietic progenitor cells (HPC) has been previously demonstrated (24). In co-cultures of cord blood-derived HPC and OP9 cells, pDC could be detected after one week, and were no longer present after three weeks in most donors (data not shown). A maximum was reached at week two, when a population (7.7±1.4%; range 0.34-20.1%) of CD123$^+$BDCA-2$^+$BDCA-4$^+$ pDC could be detected in control cultures (Figure 1A). Addition of PI3K inhibitor LY, PKB inhibitor VIII or mTOR inhibitor Rapa from day 0 reduced the cell yield at day 14 with 74±4%, 49±6% and 52±11%, respectively. In addition, continuous exposure to LY almost completely abrogated pDC development, while pDC proportions were strongly reduced by addition of VIII or Rapa (Figure 1A,B). In the absence of OP9 cells, pDC could only be detected after one week. LY, VIII and Rapa reduced pDC development during the first week both in the presence and in the absence of OP9 cells (Figure 1C), excluding the possibility that the loss of pDC was due to an effect on OP9 cells. Together, these data show a nonredundant role for PI3K-PKB-mTOR signaling during CD34-derived pDC development.

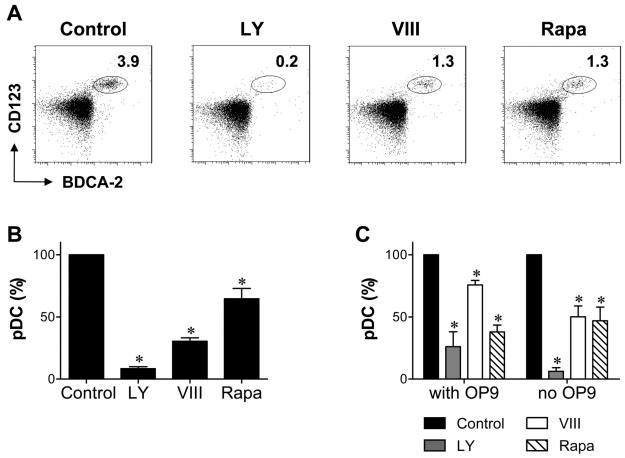

Figure 1. CD34-derived pDC development requires intact PI3K-PKB-mTOR signaling

$CD34^+$ HPC were differentiated towards pDC in the presence of LY, VIII, Rapa or their solvent DMSO. At the end of culture, pDC were identified by the expression of CD123, BDCA-2 and BDCA-4. Data are representative of at least 3 independent experiments with different donors. ***AB)*** *Co-cultures of $CD34^+$ HPC and OP9 cells were analyzed after two weeks. Representative FACS plots (A) and mean±SEM percentage pDC standardized to control (B) are shown.* ***C)*** *Cultures with and without OP9 cells were analyzed after one week. Mean±SEM percentage pDC, standardized to control, are shown. * $p<0.05$, paired Student t-test*

Inhibition of PI3K, PKB or mTOR induces apoptosis of peripheral blood pDC

The reduced pDC yield could result from inhibited differentiation, proliferation and/or survival of precursor cells, as well as from apoptosis of fully developed pDC. To investigate pDC survival regulation by PI3K-PKB-mTOR in the absence of non-pDC, which might obscure results, we used pDC isolated from peripheral blood. As demonstrated by the increased Annexin V binding in both the PI^- and the PI^+ population, addition of LY, VIII or Rapa induced apoptosis in a dose-dependent manner (Figure 2). Thus, activity of PI3K, PKB and mTOR is required for pDC survival, suggesting that the loss of pDC in inhibitor-containing differentiation cultures can at least partly be explained by the induction of apoptosis.

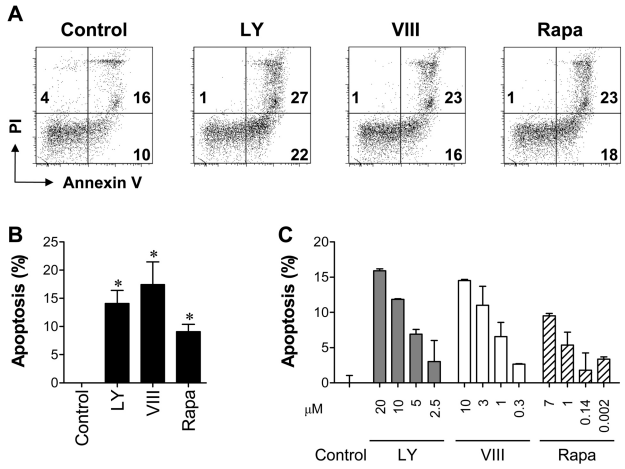

Figure 2. PI3K, PKB and mTOR regulate survival of peripheral blood pDC

Peripheral blood pDC were cultured in the presence of LY, VIII, Rapa or DMSO. ***AB)*** *Cells were analyzed for AnnexinV/PI staining after 18 hours. Apoptosis was determined as the percentage of cells staining for Annexin V and/or PI. Apoptosis was standardized to control by subtracting the percentage of apoptotic cells in control cultures from the percentage of apoptotic cells in inhibitor cultures. Shown are representative FACS plots (A) and mean±SEM apoptosis standardized to control (B) from at least 3 independent experiments with different donors.* ***C)*** *LY, VIII or Rapa was added in increasing concentrations. DMSO concentration was similar in all cultures. Apoptosis was determined by AnnexinV/PI staining after 18 hours and standardized to control as in (B). Mean±SD apoptosis in duplicate cultures is shown. * $p<0.05$, paired Student t-test*

Increased PI3K-PKB activity augments CD34-derived pDC development

The data described above show a crucial function of the PI3K-PKB-mTOR axis in human CD34-derived pDC development. To analyze whether increased signaling could promote pDC development, we increased PI3K-dependent signaling pharmacologically, through inhibition of PTEN. Addition of PTEN inhibitor VO-OHpic from day 0 increased the proportion as well as the absolute number of pDC in CD34-derived pDC differentiation cultures (Figure 3A).

In a second approach, pDC development from CD34+ HPC retrovirally transduced with a construct expressing a constitutively active form of PKB (myrPKB) was compared to pDC development from HPC transduced with a control vector. Whereas PTEN inhibition leads to increased PI3K-dependent signaling in cells in which signaling is already activated, thus augmenting endogenous PI3K activity, myrPKB ensures continuous PKB activity independent of extracellular signals. Similar to PTEN-inhibited cultures, pDC differentiation from myrPKB-expressing HPC resulted in increased pDC numbers (Figure 3B). However, cell yields of non-pDC were also increased in these cultures, resulting in a slight decrease in the proportions of pDC within the myrPKB-transduced HPC-derived population in most experiments (Figure 3B). Nevertheless, these data show that increasing PI3K-PKB signaling augments the development of pDC.

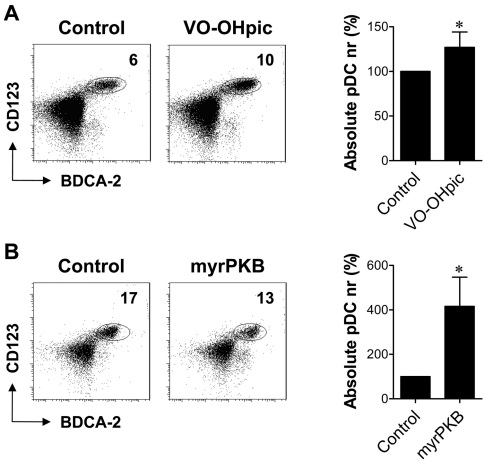

Figure 3. Increased PI3K-PKB activation improves CD34-derived pDC development

A) *CD34+ HPC were differentiated towards pDC in the presence or absence of VO-OHpic. After two weeks, cells were counted with trypan blue exclusion and analyzed for the expression of CD123, BDCA-2 and BDCA-4 by flow cytometry. Absolute numbers of pDC per well were calculated and standardized to control. Representative FACS plots showing pDC percentages and mean±SEM absolute pDC numbers are shown (n=6).* ***B)*** *CD34+ HPC, retrovirally transduced with myrPKB or a control vector, were differentiated towards pDC. The percentage of eGFP+ cells was analyzed two days after transduction to determine the transduction efficiency. After two weeks, cells were counted with trypan blue exclusion and analyzed for eGFP, CD123, BDCA-2 and BDCA-4 by flow cytometry. Absolute numbers of eGFP+ pDC per well were determined, corrected for the difference in transduction efficiency and standardized to control. Representative FACS plots showing pDC within the eGFP+ population and mean±SEM absolute eGFP+ pDC numbers are shown (n=6). * p<0.05, Wilcoxon signed rank test*

Ectopic PKB activation promotes costimulatory molecule expression

To assess the functional characteristics of pDC generated through increased PKB activity, we evaluated MHC class II and costimulatory molecule expression. Without stimulation, pDC isolated from peripheral blood express HLA-DR, but show low to undetectable expression of CD40 and CD86 (25). A similar pattern was observed for unstimulated control-transduced CD34-derived pDC (Figure 4). Expression was upregulated by stimulation with the synthetic TLR7 and TLR9 ligands Lox and CpG. MyrPKB increased CpG- and Lox-induced HLA-DR, CD40 and CD86 expression (Figure 4). In addition, the basal expression of HLA-DR and CD86 was enhanced in pDC expressing myrPKB (Figure 4), suggesting a generally improved ability to express these molecules rather than an effect on TLR-induced upregulation. Thus, in addition to promoting pDC development, improved PI3K-PKB activity supports the expression of MHC class II and costimulatory molecules by the resulting pDC.

Constitutive PKB activation increases CD34-derived pDC development in vivo

To investigate the effect of increased PKB activity on in vivo CD34-derived pDC development, myrPKB- or control-transduced human CD34+ HPC were transplanted into β2-microglobulin$^{-/-}$NOD/SCID mice. Six weeks post-transplantation, human pDC originating from transduced HPC could be recognized in the bone marrow by the expression of eGFP, CD123 and BDCA-2. In accordance with the increased pDC numbers observed in vitro, mice transplanted with myrPKB-transduced HPC showed 8-fold increased pDC percentages within the eGFP+ population compared to mice that had received a transplant of control-transduced

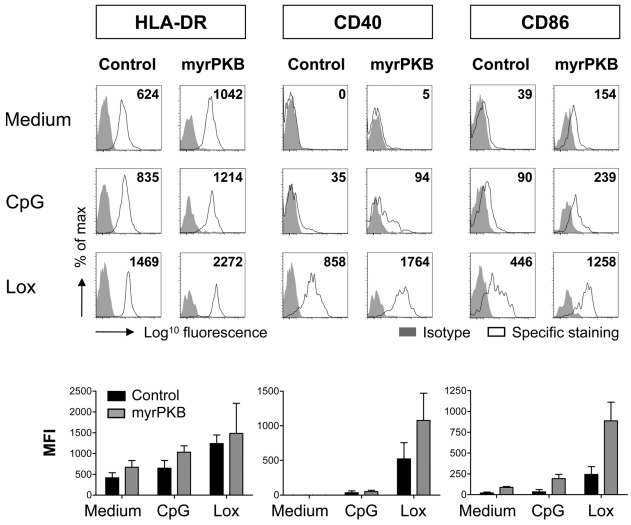

Figure 4. Ectopic PKB activation increases costimulatory molecule expression by pDC

$CD34^+$ HPC, retrovirally transduced with myrPKB or a control vector, were differentiated towards pDC in two weeks. Cells were either left unstimulated, or CpG or Lox were added during the last 18 hours of culture. Expression of HLA-DR, CD40 and CD86 by $eGFP^+CD123^+BDCA\text{-}2^+BDCA\text{-}4^+$ pDC was determined. Specific staining was calculated by subtracting the mean fluorescence intensity (MFI) of the isotype control from the MFI measured for each marker. Representative FACS plots and mean±SEM specific staining are shown (n=3). Numbers in the FACS plots indicate the specific staining calculated for the experiment shown.

HPC (Figure 5A). In addition, expression of CD86 by these pDC was enhanced 16-fold (Figure 5B). These data confirm the important role of PI3K-PKB-mTOR signaling in CD34-derived pDC development, and show that augmented signaling promotes the development of pDC with an increased activation status.

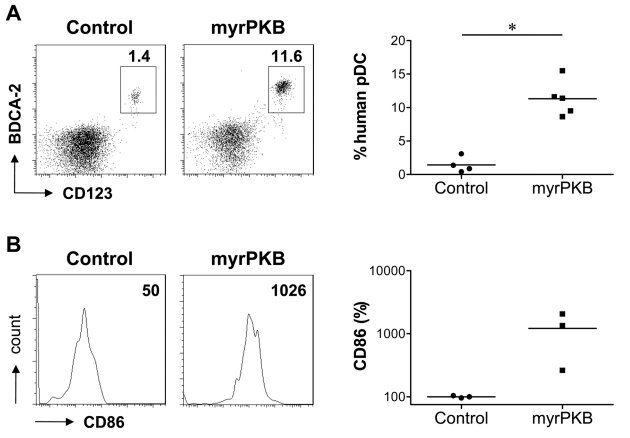

Figure 5. Constitutively active PKB increases development and activation status of CD34-derived pDC in vivo

*$CD34^+$ HPC were retrovirally transduced with myrPKB or a control vector. Day 3 unsorted cells were intravenously injected into β2-microglobulin$^{-/-}$ NOD/SCID mice. **A)** Six weeks post-transplantation, the bone marrow was analyzed for the presence of $CD123^+BDCA\text{-}2^+$ human pDC within the $eGFP^+$ fraction. Shown are representative FACS plots gated on $eGFP^+$ cells and percentage $CD123^+BDCA\text{-}2^+$ human pDC within the $eGFP^+$ population per mouse (Control: n=4; myrPKB: n=5). **B)** CD86 expression by human pDC in different experiments was determined and standardized to control per experiment. Shown are representative FACS plots gated on $eGFP^+CD123^+BDCA\text{-}2^+$ human pDC, and standardized CD86 expression by human pDC per mouse (Control: n=3; myrPKB: n=3). * $p<0.05$, Mann-Whitney U test*

Improved cytokine production following enhanced PI3K-PKB activity

An important functional characteristic of pDC is the secretion of inflammatory cytokines, of which IFN-α is the most pronounced. Specific analysis of cytokine production by transduced in vitro generated CD34-derived pDC required analysis by intracellular cytokine staining, which allowed gating on $eGFP^+CD123^+BDCA\text{-}2^+$ pDC. However, although IFN-α could be measured in the supernatants of cultures stimulated overnight with Lox or CpG, we were unable to show IFN-α^+ pDC by 5 hour intracellular cytokine staining (data not shown). To enable analysis of IFN-α production we made use of HSV, a DNA virus that activates pDC via both TLR9-dependent and -independent pathways (26). MyrPKB-transduced pDC showed an increased ability to produce IFN-α following HSV stimulation compared

to control-transduced cells (Figure 6A,B). Not only the percentage of IFN-α$^+$ pDC, but also the IFN-α staining intensity within the IFN-α$^+$-pDC population was increased (Figure 6B), suggesting enhanced IFN-α production per producing pDC. In addition, improved Lox- and CpG-induced IFN-α production was demonstrated for peripheral blood pDC pre-incubated with VO-OHpic to enhance PI3K-mediated PKB activation (Figure 6C). Interestingly, HSV- and Lox-induced TNF-α production by myrPKB-transduced CD34-derived pDC was also increased (Figure 6D,E). Together, these data show that increasing activity of the PI3K-PKB axis may be helpful to promote inflammatory cytokine production by pDC activated through TLR7 or TLR9 ligands.

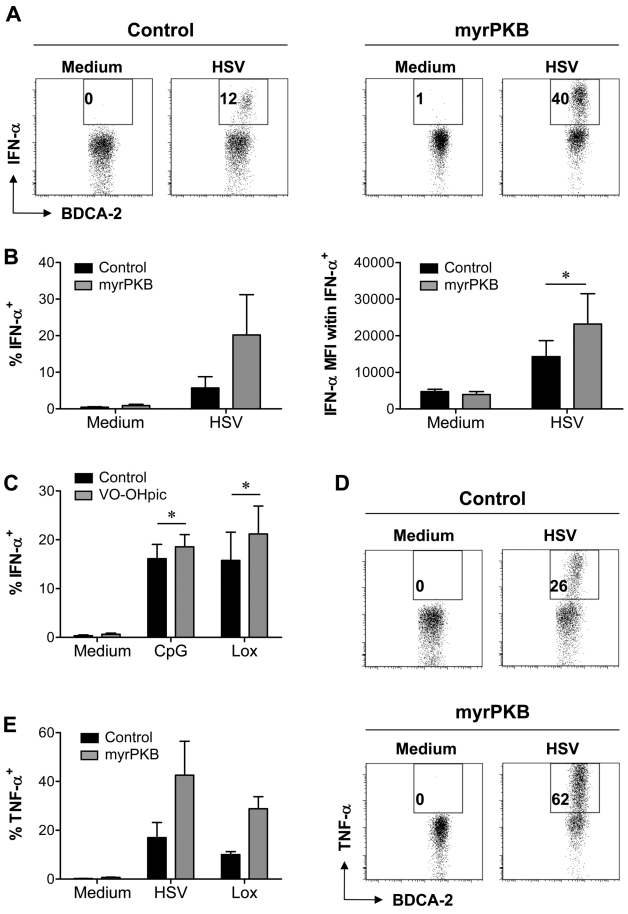

Figure 6. Augmented IFN-α and TNF-α production by pDC with increased PI3K-PKB activity

AB) *CD34$^+$ HPC, retrovirally transduced with myrPKB or a control vector, were differentiated towards pDC in two weeks. Cells were either left unstimulated or HSV was added during the last 5 hours of culture. The percentage of eGFP$^+$CD123$^+$BDCA-2$^+$ pDC producing IFN-α was determined by intracellular cytokine staining. The IFN-α staining intensity (MFI) on eGFP$^+$IFN-α$^+$ pDC was determined. Representative FACS plots showing eGFP$^+$ pDC (A), and mean±SEM percentage IFN-α producing pDC and IFN-α staining intensity within the IFN-α$^+$ pDC (B) are shown (n=3).* ***C)*** *Peripheral blood pDC were cultured in the presence or absence of VO-OHpic. After overnight pre-incubation, pDC were cultured for 5 hours with medium, CpG or Lox. Mean±SEM percentage IFN-α producing pDC of at least 4 independent experiments with different donors is shown.* ***DE)*** *CD34$^+$ HPC were cultured as for (AB). Cells were left without stimulus, or HSV or Lox was added during the last 5 hours of culture. The percentage of eGFP$^+$CD123$^+$BDCA-2$^+$ pDC producing TNF-α was determined by intracellular cytokine staining. Representative FACS plots showing eGFP$^+$ pDC (D) and mean±SEM percentage TNF-α producing pDC (E) are shown (n=3). * p<0.05, paired Student t-test*

Decreased TLR-induced PI3K-PKB-mTOR-S6K activity in pDC from chronic HBV patients

Considering the regulatory role of PI3K-PKB-mTOR in pDC function combined with the impaired pDC functionality described for patients chronically infected with HBV (25), we compared the CpG- and Lox-induced activity of this signaling module in HBV patients and healthy individuals. Since in particular pDC derived from patients positive for HBeAg (HBe^{pos}) display a functional defect (25), HBe^{pos} and HBe^{neg} patient pDC were analyzed separately. S6 phosphorylation, which is induced by mTOR-activated S6K, was used as readout for

PI3K-PKB-mTOR-S6K signaling. Phosphorylation in unstimulated pDC from HBe^{pos} patients was lower (24±8% decreased) than in pDC from healthy subjects (Figure 7A). Whereas CpG or Lox stimulation resulted in a time-dependent phosphorylation of S6 in pDC from healthy subjects, no difference was found between HBe^{pos}-derived pDC cultured in the presence or absence of TLR ligands (Figure 7A,B). Accordingly, compared to healthy subject pDC, S6 phosphorylation was reduced in CpG- or Lox-stimulated HBe^{pos} patient pDC (Figure 7C). pDC from HBe^{neg} patients appeared unaffected, showing S6 phosphorylation comparable to healthy control pDC (Figure 7C). Similar results were obtained for the other time points (data not shown). These data demonstrate reduced activation-induced PI3K-PKB-mTOR-S6K signaling in the functionally impaired pDC of HBe^{pos} HBV-infected patients.

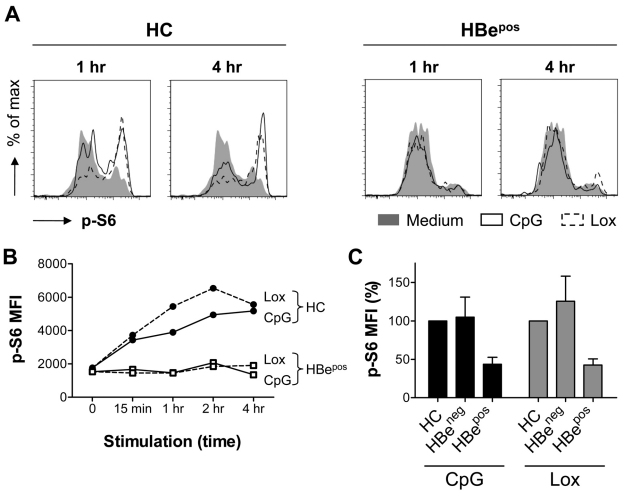

Figure 7. Decreased TLR-induced S6 phosphorylation in pDC from HBe^{pos} chronic HBV-infected patients

PBMC from healthy subjects, HBe^{pos}- and HBe^{neg}-HBV infected patients were cultured for 5 hours. CpG or Lox were added for the indicated periods. p-S6 staining intensity was determined within the CD11c^{-}BDCA-4^{+} pDC population. Results are representative for 3 subjects per group. ***A)*** *FACS plots show p-S6 staining of unstimulated, CpG- and Lox-stimulated pDC from a representative HBe^{pos} patient and an age and gender matched healthy control (HC).* ***B)*** *p-S6 staining intensities (MFI) from a representative HBe^{pos} patient and matched HC are shown.* ***C)*** *p-S6 MFIs measured in 1 hour stimulated cultures were standardized to HC. Mean±SEM MFI are shown.*

Discussion

Despite the key function pDC have in immunity, the current knowledge on methodologies to manipulate their development or function remains limited. The present study identified the PI3K-PKB-mTOR axis as a possible target. The generation and survival of human pDC were dependent on PI3K-PKB-mTOR signaling, and pDC from chronic HBV infected patients, which are functionally impaired, demonstrated reduced PI3K-PKB-mTOR-S6K activity. Importantly, our data demonstrate that enhanced activation of this pathway improves human pDC development and function. Both in vitro and in vivo, PKB hyperactivation resulted in increased pDC numbers, augmented MHC class II and costimulatory molecule expression, and enhanced production of cytokines including IFN-α.

Inhibition of PI3K, PKB or mTOR during human CD34-derived pDC development resulted in significantly reduced pDC yields, demonstrating a nonredundant role for the PI3K-PKB-mTOR signaling module. We and others have reported a critical function for this pathway in modulating the homeostasis of other human DC subsets, which were generated in decreased numbers in the presence of LY or Rapa (16, 19-21). Whereas in those studies differentiation of CD34- and monocyte-derived myeloid DC appeared normal except for the reduced yields, here we show decreased pDC proportions due to PKB or mTOR inhibition and almost completely abolished pDC development in the presence of LY. The disadvantage of pDC compared to other cells generated in these cultures could reflect inhibited differentiation. However, reduced survival during or after differentiation could also contribute, as shown

by the inhibitor-induced apoptosis of peripheral blood pDC. In contrast to human myeloid DC, in vitro models exclusively generating human pDC are lacking, precluding separate investigation of precursor proliferation, survival and differentiation. The severely reduced expansion observed here suggests a role for the PI3K-PKB-mTOR pathway in proliferation, which would be in accordance with the function of this signaling module described for myeloid DC progenitors (19). In line with the inhibition of DC observed in rapamycin-treated mice (27), the PI3K-PKB-mTOR dependency of human myeloid DC described previously (16, 19-21) and of pDC reported here together suggest a central function of this pathway in human DC development.

The finding that mTOR activity is essential for efficient pDC development and survival appears relevant when considering the therapeutic applications of rapamycin. Inhibited pDC immunity could contribute to immunosuppression when rapamycin is applied to prevent transplant rejection, while the same feature may counteract the desired consequences when it is used as an anti-cancer drug (28-29). However, tumor-infiltrating pDC are often believed to become tolerogenic in response to tumor-secreted factors (30-33). In that light, pDC loss may actually be beneficial.

PI3K-PKB-mTOR signaling appeared not only essential but also rate-limiting during development of human pDC. Introduction of a constitutively active PKB mutant augmented the in vitro yields of pDC as well as non-pDC, whereas pharmacological inhibition of PTEN specifically promoted pDC numbers resulting in increased pDC percentages. This discrepancy is likely explained by the fact that myrPKB ensures PKB-dependent signaling in all cells, whereas enhancing PKB activity through PTEN inhibition requires Flt3L-induced PI3K activation, which is restricted to the DC lineage (13). In line, the unchanged murine pDC numbers found in vivo following PTEN deletion have been suggested to result from limited endogenous activation of this pathway (13). The strongly increased pDC development from myrPKB-transduced human progenitors found not only in vitro but also in vivo appears interesting. Promoting activation of this signaling module could be used to increase pDC numbers in a therapeutic setting.

Besides enhancing their numbers, increased PI3K-PKB activity promoted the expression of MHC class II and costimulatory molecules by pDC. Increased TLR-induced upregulation could be responsible for the high expression in TLR-stimulated cells, but the enhanced expression by unstimulated pDC suggests an activation-independent effect. Moreover, treatment of peripheral blood pDC with VO-OHpic during CpG- or Lox-stimulation had no effect, whereas CD34-derived pDC differentiated in the presence of VO-OHpic showed a modestly increased expression (data not shown). Evidently, the use of different models and experimental strategies, including manipulating of signaling during or after development and using heterogeneous or homogeneous cell populations, will affect the outcome of the experiment. However, on the basis of our data, the enhanced expression of MHC class II and costimulatory molecules appears cell-intrinsic. Since the PI3K-PKB-mTOR axis is an important regulator of protein translation (34) and PI3K-dependent protein synthesis has been implicated in costimulatory molecule upregulation by LPS-activated murine bone marrow-derived DC (35), PKB-mediated translation might be involved in this process.

Although IFN-α production was also increased by PKB hyperactivation, its induction required an activation signal regardless of PI3K-PKB activity. This is in accordance with the observation that S6K, a kinase activated by mTORC1, promotes TLR9-induced IFN-α secretion by stabilizing the TLR9-MyD88 complex (12), indicating that the PI3K-PKB-mTOR signaling module can enhance but not initiate IFN-α secretion. HSV-induced IFN-α secretion can be TLR9 dependent and -independent, whereas CpG-mediated pDC activation is completely TLR9-dependent. We were unable to investigate CpG-induced IFN-α production in CD34-derived pDC due to technical difficulties, but IFN-α production by CpG-stimulated

peripheral blood pDC was increased by enhanced activation of the PI3K-PKB axis. In addition, PKB hyperactivation increased Lox-induced IFN-α production, suggesting that TLR7-mediated cytokine production also profits from PI3K-PKB-mTOR signaling. Besides promoting stabilization of the TLR-MyD88 complex, alternative explanations exist. These include a general increased mRNA translation due to high PKB activity (34), or effects on TLR expression. Finally, reduced CpG uptake and CpG-TLR9 colocalization has been reported following PI3K-inhibition (36), indicating a possible contribution of these processes.
Although its production is induced through NF-κB activation rather than the PI3K-PKB-mTOR signaling module, TNF-α production was improved by increasing PKB activity. Besides the mechanisms suggested above for IFN-α, an additional explanation for increased TNF-α production following PKB hyperactivation may lie in PKB-mediated NF-κB activation (37). In line with our data, inhibition of mTOR has been shown to inhibit CpG-induced TNF-α and IL-6 secretion in addition to IFN-α (12). Thus, although the exact mechanisms remain to be determined, it is clear that pDC ability to produce IFN-α and TNF-α is promoted by enhanced PKB activity.
As discussed above, the enhanced activation of PKB results in increased pDC numbers, augmented MHC class II and costimulatory molecule expression, and improved IFN-α and TNF-α production. Development of therapies that induce pDC with improved functionality could be used to treat tumors or persistent infections evading immunity. As demonstrated here, pDC from HBeAg-positive chronic HBV-infected patients, which have a severely reduced ability to produce IFN-α (25), show impaired activation-induced phosphorylation of S6, indicating ineffective PI3K-PKB-mTOR-S6K signaling. Furthermore, the reduced activity of this pathway found in unstimulated pDC could be related to the decreased pDC numbers present in these patients (10). Therapies aiming to enhance activity of this signaling module might have favorable effects on anti-HBV immunity. However, potential side effects such as the risk of autoimmunity due to dysregulated pDC activation have to be taken into account (38-41). In addition, since PKB regulates many different processes in a large variety of cell types, careful targeting is required. However, similar strategies have been applied to promote the functionality of murine and human myeloid DC without significant side effects (18, 42-43), indicating that with careful consideration of the disease and potential side effects, the PI3K-PKB-mTOR axis may provide a useful therapeutic target.
In conclusion, intact PI3K-PKB-mTOR signaling is required for human pDC development and survival. Increasing signaling along this pathway increases not only pDC development, but also their activation status, enhancing the expression of MHC class II and costimulatory molecules, and promoting the production of cytokines. This knowledge may help in understanding the consequences of specific drugs and may contribute to the development of novel treatment strategies aiming to interfere with the human immune system. Manipulation of the PI3K-PKB-mTOR signaling module or its downstream targets could be used to improve the generation and function of pDC in order to augment immunity.

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7

GENERAL DISCUSSION

Introduction

Dendritic cells (DC) can induce immunity as well as tolerance. The type of immune response initiated by a DC is determined by the subset to which the DC belongs, its lifespan and its maturation state. These characteristics are regulated by environmental signals, which are translated into cellular actions by intracellular signal transduction pathways, effector proteins and transcription factors. Novel insights in the molecular regulation of DC subset development, survival and function increase our understanding of DC biology under different pathophysiological conditions and may enable the development of novel treatment strategies to manipulate immunity. The work described in this thesis has evaluated the role of PI3K-PKB-mTOR, canonical NF-κB and JAK2/STAT5 in the regulation of human DC biology. Chapters 3-5 focused on myeloid DC, including interstitial DC, Langerhans cells and monocyte-derived DC, while Chapter 6 focused on plasmacytoid DC.

Dendritic cell development

From uncommitted progenitor to subset-specific precursor

Similar to all other hematopoietic lineages, the DC lineage is derived from uncommitted self-renewing stem cells, which give rise to progenitors with gradually restricted developmental options. The differentiation and maintenance of DC restricted progenitors is regulated by Flt3 ligand (Flt3L), GM-CSF and M-CSF (1-2). One action of these cytokines is the activation of phosphatidylinositol 3-kinase (PI3K), which in turn activates protein kinase B (PKB/c-AKT) and several downstream substrates such as mammalian target of rapamycin (mTOR) (3-4). A regulatory role for the PI3K-PKB-mTOR pathway is supported by the fact that inhibition of mTOR by in vivo administration of rapamycin severely reduces DC numbers in murine spleens and bone marrow (5). Although circulating myeloid DC were unaffected in kidney transplant patients treated with rapamycin (6), in vitro yields of human CD34-derived plasmacytoid DC (Chapter 6), interstitial DC (Chapter 3), Langerhans cells (Chapter 3) and monocyte-derived DC (6-8) were all severely reduced following pharmacological inhibition of PI3K, PKB or mTOR. The broad effects across several DC lineages could reflect a widespread function in all individual subsets, a suggestion supported by the multiple cellular processes this signaling module controls in a wide range of hematopoietic cells (9). Additionally, regulation of early multipotent progenitors could explain multi-lineage effects. In Chapter 3 we demonstrated PI3K-PKB-mTOR-dependent proliferation and survival of early myeloid DC progenitors, and control of progenitor expansion in CD34-derived plasmacytoid DC cultures was shown in Chapter 6. These data indicate that cytokine-induced PI3K-PKB-mTOR signaling regulates the maintenance of early DC progenitors (Figure 1). The contributions of other candidate signaling modules, such as MAPK or JAK/STAT which are activated by Flt3L and GM-CSF and are known to regulate proliferation (10-11), remain to be defined for DC progenitors. The reduced progenitor expansion in CD34-derived myeloid DC cultures treated with NF-κB inhibitors described in Chapter 4 indicates that canonical NF-κB transcription factors could also be involved.

Besides the maintenance and expansion of early progenitors, efficient DC development requires commitment to a specific DC lineage and the initiation of differentiation. Transcription factor STAT3, which is activated by Flt3L and GM-CSF, appears both required and instructive in DC commitment of early hematopoietic progenitors (12-13) (Figure 1). Interestingly, inhibition of TLR-induced mTOR activity resulted in reduced STAT3 phosphorylation and activity (14), suggesting that PI3K-PKB-mTOR signaling may be able to promote activation of STAT3, and thus DC commitment. However, the importance of this signaling cascade in

DC differentiation is debatable. In vitro differentiation of CD34-derived myeloid pre-DC was observed regardless of PI3K or mTOR inhibition, albeit in reduced numbers (Chapter 3). In addition, plasmacytoid DC could be retrieved in cultures treated with PKB or mTOR inhibitors (Chapter 6), indicating that differentiation capacity of early progenitors was not completely abolished. The reduced plasmacytoid DC proportions found in these cultures and the almost complete loss of plasmacytoid DC in PI3K inhibited cultures could indicate reduced differentiation, but could also result from specific apoptosis (Chapter 6). Because the role of PI3K-PKB-mTOR in DC differentiation has not been conclusively proven, the relevance and possible implications of the suggested interaction between mTOR and STAT3 for DC commitment remain to be determined. Furthermore, the consequences of STAT3 regulation by other Flt3L- or GM-CSF-activated signaling modules are yet to be investigated.

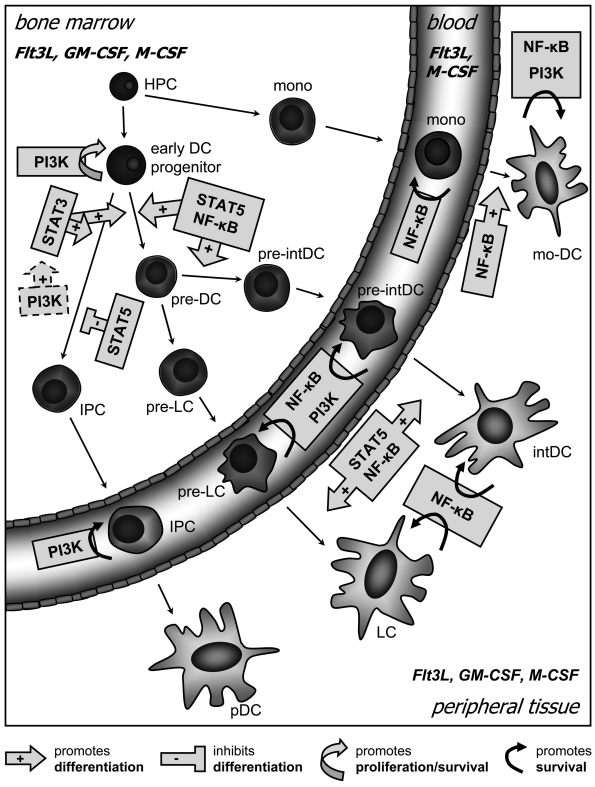

Figure 1. Integrated model on human DC subset homeostasis

DC development is initiated in the bone marrow, where uncommitted hematopoietic progenitor cells (HPC) give rise to monocytes (mono) and early DC progenitors. Maintenance and expansion of these early progenitors is mainly mediated through PI3K-PKB-mTOR (PI3K) signaling. DC differentiation and lineage commitment is mediated through intracellular signaling modules and transcription factors that are differentially regulated by various important cytokines including Flt3L and GM-CSF. Whereas Flt3L-mediated activation of STAT3 supports DC differentiation in general, GM-CSF-induced STAT5 activation affects DC subset differentiation. Subset-specific pre-DC, interferon producing cells (IPC) and monocytes egress from the bone marrow to enter the circulation, where they are sustained by M-CSF and Flt3L. GM-CSF could contribute under inflammatory circumstances. Survival within the circulation is mediated through activation of PI3K-PKB-mTOR signaling as well as NF-κB transcription factors. Terminal differentiation occurs during or after tissue infiltration, and is dependent on GM-CSF besides Flt3L. Activation of STAT5 and canonical NF-κB has been implicated in GM-CSF-induced terminal differentiation, and increased STAT5 activity contributes to enhanced DC differentiation at inflammatory sites. The signaling modules regulating survival of terminally differentiated DC differ from those regulating pre-DC survival. Interstitial DC and Langerhans cells become independent of PI3K-PKB-mTOR signaling, whereas the survival of monocyte-derived DC requires signaling along this pathway. Canonical NF-κB transcription factors seem to be required for the survival of interstitial DC, Langerhans cells and monocyte-derived DC.

Abbreviations: HPC, hematopoietic progenitor cell; mono, monocyte; IPC, interferon producing cell; pre-DC, precursor DC; intDC, interstitial DC; LC, Langerhans cell; mo-DC, monocyte-derived DC; pDC, plasmacytoid DC; PI3K, short for the PI3K-PKB-mTOR signaling module

PU.1 is another transcription factor critical for DC differentiation (15). However, its expression is not restricted to the DC lineage, and it has been suggested that specific PU.1 expression levels induce differentiation of distinct hematopoietic lineages (16-17). In accordance with the idea that cell fate decisions depend on the concentration of PU.1, its expression is

regulated by a wide range of cytokines. As may be expected considering its crucial function in DC development, Flt3L induces PU.1 expression (12). Surprisingly, GM-CSF may reduce PU.1 expression through activation of STAT5, although this conclusion is primarily based on experiments with supraphysiological levels of STAT5 activity (Chapter 5). Besides promoting DC development in general, PU.1 expression levels also affect lineage decisions within the DC population, as demonstrated by the enhanced Langerhans cell commitment of human $CD34^+$ progenitors with increased PU.1 expression (18). TGF-β reduces STAT5 activity and increases PU.1 expression, a function that could contribute to TGF-β-induced Langerhans cell development (Chapter 5). Taken together, it appears that specific cytokines regulate PU.1 expression through distinct mechanisms, including the regulation of STAT5 activity. These pleiotropic mechanisms may be exploited to precisely adjust DC subset development to different pathophysiological conditions.

Additional signaling events can also contribute to DC subset specification. STAT5 inhibits plasmacytoid DC commitment of murine CDP by inhibiting transcription of interferon regulatory factor 8 (IRF8), a transcription factor required for plasmayctoid DC development (19) (Figure 1). Furthermore, activation of both STAT5 and canonical NF-κB transcription factors has been associated with increased expression of the transcription factor IRF4, which is primarily involved in the development of non-plasmacytoid DC lineages (Chapter 2). Subset-specific regulators of the human myeloid lineages include noncanonical NF-κB protein RelB, which is required for interstitial DC but not Langerhans cell development (20). Similarly, development of pre-interstitial DC depends more strongly on canonical NF-κB transcription factor activity than pre-Langerhans cell differentiation (Chapter 4), and whereas STAT5 impedes commitment to the Langerhans cell lineage, its activity is required to induce pre-interstitial DC development (Chapter 5) (Figure 1). Since these signaling proteins are activated by GM-CSF, these data could indicate GM-CSF-dependent subset regulation. However, the unchanged plasmacytoid DC numbers in $GM\text{-}CSF^{-/-}$ mice suggests that, at least under steady state conditions, the in vivo contribution of GM-CSF to subset-specification may be limited (21). Instead, this is achieved by other factors likely acting on the signaling proteins mentioned above. While the limited role of GM-CSF in the steady state is unsurprising considering its low concentration, GM-CSF-mediated effects likely increase under inflammatory conditions, thereby providing a mechanism to adjust DC subset development to environmental demands.

From subset-specific precursor to dendritic cell

After egress from the bone marrow, circulating pre-DC and interferon producing cells (IPC) may respond to Flt3L or M-CSF, whereas Flt3-negative monocytes are thought to be maintained by M-CSF (1-2). Circulating cells of the DC lineage are all precursors, lacking the typical veiled cell morphology characteristic for fully differentiated DC (22). Since in-blood DC differentiation appears absent under steady state conditions, the main function of circulating growth factors acting on DC precursors is regulation of their maintenance. As shown in Chapters 3 and 6, pre-interstitial DC, pre-Langerhans cells and IPC depend on PI3K-PKB-mTOR signaling for their survival (Figure 1). Furthermore, canonical NF-κB activity is required for the survival of monocytes, pre-interstitial DC and pre-Langerhans cells (Chapter 4) (Figure 1). Pharmacological inhibition of MEK/ERK during monocyte-derived DC differentiation also reduces DC yields (8, 23), suggesting that this signaling module mediates the survival of either monocytes or differentiated monocyte-derived DC. Together, these signaling modules and possibly others ensure the maintenance of circulating DC precursors until they infiltrate peripheral tissues.

The high turnover of DC precursors in the blood indicates rapid tissue infiltration (24). Differentiation is induced during or after migration. In lymphoid tissue, Flt3L may cause some

proliferation in addition to driving differentiation of pre-DC and IPC (1), but the signaling modules responsible for this, especially for human DC, are as yet undefined. Nonlymphoid tissues are likely infiltrated by pre-DC, IPC and monocytes (1, 25). As demonstrated by the loss of DC in gut and skin of mice deficient for the GM-CSF receptor (GM-CSFR) or GM-CSF (21, 26-27), locally produced GM-CSF contributes to DC differentiation at these sites. Indeed, differentiation of human CD34-derived interstitial DC and Langerhans cells from their respective pre-DC depends on the activity of canonical NF-κB and STAT5 (Chapters 4 and 5), factors activated by GM-CSF (Figure 1). In line, DC differentiation from monocytes requires canonical NF-κB activity (Chapter 4) (Figure 1). Despite the crucial function of STAT3 in initiating DC differentiation at the early progenitor stage, its role in terminal DC differentiation in nonlymphoid tissue has not been investigated. Its redundancy in GM-CSF-driven cultures (19, 28) could indicate that STAT3 might not be essential for GM-CSF-driven differentiation at peripheral sites. As described above, the PI3K-PKB-mTOR signaling module appears to regulate survival and proliferation rather than differentiation (Chapter 3). Interestingly however, activity of glycogen synthase kinase-3β (GSK-3β), which is negatively regulated by PKB-dependent phosphorylation, is required to avoid monocyte-to-macrophage differentiation in monocyte-derived DC differentiation cultures (29-30). Increasing PKB activity in monocytes results in their differentiation towards macrophages rather than DC, indicating that despite its apparent redundancy, PKB may still affect differentiation of certain DC subsets.

The consequences of inflammation on dendritic cell development

Inflammation not only increases the rate of DC development, but also changes the composition of the DC pool. It triggers migration of peripheral tissue DC to draining lymph nodes and necessitates generation and attraction of additional DC to the inflammatory site (1, 25). The contribution of monocyte-derived DC increases in this setting. Monocytes are attracted in large numbers in a CCR2-dependent manner, and the high levels of GM-CSF at the inflammatory sites they infiltrate support their differentiation towards DC (31-33). In addition, the altered cytokine milieu may enhance differentiation of infiltrating pre-DC and IPC. Terminal differentiation of pre-DC is improved by conditionally increasing STAT5 activity (Chapter 5), a state that will presumably be induced by the high GM-CSF concentrations present at sites of inflammation. Although yet undetermined, it is also very likely that STAT5 is involved in monocyte-to-DC differentiation. Besides high STAT5 activity, increased activation of canonical NF-κB transcription factors and possibly MEK/ERK signaling will most likely contribute to enhanced DC differentiation in inflamed tissue. In addition to GM-CSF and M-CSF-induced signaling, inflammation-induced production of additional cytokines such as TNF-α, IL-4 and TGF-β could manipulate DC differentiation (1), while the presence of TLR ligands may provide a short-cut from usual differentiation steps. TLR-induced signaling, including canonical NF-κB transcription factor activation, induces very rapid DC differentiation of monocytes, IPC and even early progenitor cells (34-37). Finally, the increased concentrations of Flt3L present during inflammation can also support DC differentiation. However, Flt3L-induced differentiation yields DC with a relatively tolerogenic function (Chapter 2) that may be involved in restoring tolerance rather than promoting immunity.

Next to alterations in local DC differentiation, changes in the cytokine profiles in blood and bone marrow influence the development of progenitors and precursors. Systemically elevated Flt3L levels induce expansion of early bone marrow progenitors through activation of PI3K-PKB-mTOR signaling, while activation of STAT3 and PU.1 results in DC differentiation. The simultaneous increase in GM-CSF promotes development of more immunogenic DC (Chapter 2), and could also affect DC subset decisions as explained above. Besides

GM-CSF-STAT5-mediated inhibition of plasmacytoid DC commitment, the examples of signaling-driven subset-specification given above include regulation of the human interstitial DC and Langerhans cell lineages. The short lifespan and constant replenishment from blood-borne precursors is undisputed for almost all DC subsets. However, Langerhans cells have been suggested to be long-lived, maintained independently from circulating precursors in the steady state and replenished from monocytes in an M-CSF receptor-dependent manner under inflammatory conditions (38-40). Although this hypothesis has not been conclusively proven, in particular for human Langerhans cells, its confirmation would challenge the relevance of the subset-specification mechanisms described above. It appears likely however, that similar processes are involved in the regulation of other subset-specifications. Taken together, inflammation-induced changes in systemic and local cytokine profiles and the resulting signaling in bone marrow, circulating and local precursors allows adaptation of DC development to situation-specific requirements.

Dendritic cell survival

The lifespan of a DC significantly influences its function in immunity. In contrast to lymphoid-resident DC, which have an estimated half-life of approximately two days, under steady state conditions migratory DC reside in peripheral tissue for periods ranging from one to four weeks (25, 41). Considering this relatively long residence combined with the rather limited availability of GM-CSF in non-inflamed tissue, the independence of GM-CSF demonstrated for terminally differentiated CD34-derived interstitial DC and Langerhans cells appears appropriate (Chapter 3-Appendix). In contrast, human monocyte-derived DC (7) and murine bone marrow-derived DC (42) die rapidly following GM-CSF deprivation. These DC, which are generated in vitro through culture with GM-CSF and IL-4, likely mirror DC differentiated in inflamed tissues. Since such DC encounter high GM-CSF concentrations and migrate away rapidly towards the draining lymph nodes, a decrease in GM-CSF-dependency might not be required. Site-specific signals inducing adjustment to the environment during DC differentiation may account for the differences in survival regulation observed for specific DC subtypes.

In correlation with the reduced reliance on GM-CSF, a decrease in GM-CSFR expression was observed in CD34-derived myeloid DC at the end of differentiation (43). Moreover, the intrinsic resistance to apoptosis of these cells may be generally high. In response to cellular stress such as growth factor deprivation, apoptosis is initiated through the mitochondrial pathway to caspase activation, which requires expression of APAF-1 (41, 44-45). Thus, the low APAF-1 levels in terminally differentiated myeloid DC (Chapter 3-Appendix) might hinder activation of effector caspases through this pathway. In addition, the highly increased mRNA expression of anti-apoptotic IAP1 and reduced levels of IAP-inhibitor DIABLO invite further investigation, even though the high IAP1 expression could not yet be confirmed at the protein level (Chapter 3) (46-47). Finally, besides a potential reduction in caspase-dependent mitochondria-induced apoptosis, terminally differentiated myeloid DC also express reduced levels of AIF mRNA (Chapter 3), a mitochondrial factor that induces cell death in a caspase-independent manner (48). Interestingly, while the sensitivity to mitochondria-induced apoptosis likely decreases during differentiation of CD34-derived myeloid DC, APAF-1 expression increases during monocyte-to-DC differentiation (Figure 2). Although the significance of these findings remains to be proven, cell-intrinsic differences in sensitivity to mitochondria-mediated apoptosis could explain the growth factor dependency of distinct DC subsets.

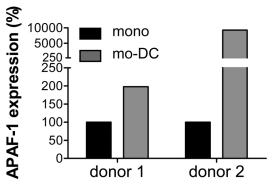

Figure 2. APAF-1 expression increases during monocyte-to-DC differentiation
Monocytes (mono) were differentiated towards monocyte-derived DC (mo-DC) by 6-day culture in the presence of 10 ng/ml GM-CSF and 10 ng/ml IL-4. Total cell lysates of monocytes and mo-DC were produced in laemmli sample buffer, protein concentrations were determined by Lowry method and equal amounts of protein were separated on 8% SDS-PAGE. Expression of APAF-1 was determined, quantified and standardized to expression in monocytes. Two independent experiments with different donors are shown.

In addition to general differences in apoptosis resistance, distinct signal transduction pathways may regulate survival of specific DC subsets. To prevent apoptosis of monocyte-derived DC, GM-CSF-induced PI3K-PKB-mTOR signaling promotes the expression of Mcl-1 (23), a member of the anti-apoptotic Bcl-2 protein family that is controlled by mTOR at the translational level (49). CD34-derived pre-DC are also dependent on PI3K-PKB-mTOR, and show a high expression of Mcl-1 (Chapter 3 and Figure 3). In contrast, Mcl-1 mRNA and protein expression is reduced in terminally differentiated interstitial DC and Langerhans cells, which is associated with a reduced dependency on PI3K-PKB-mTOR signaling (Chapter 3 and Figure 3). Considering the association between Mcl-1 expression and PI3K-PKB-mTOR dependency, it is tempting to speculate that the expression of mTOR-regulated survival proteins by specific DC subsets may provide an indication whether their survival is dependent on PI3K-PKB-mTOR signaling. Another mTOR-dependent Bcl-2 family member is Bcl-XL (50), an anti-apoptotic protein that is regarded a crucial molecular target in DC survival regulation (41). Similar to Mcl-1, Bcl-XL is expressed at relatively low levels in terminally differentiated myeloid DC (Chapter 3). In contrast, plasmacytoid DC, which are dependent on PI3K-PKB-mTOR-mediated survival signaling (Chapter 6), express high levels of Bcl-XL (51). The expression of Mcl-1 and Bcl-XL may provide a possible molecular explanation for the distinct contribution of the PI3K-PKB-mTOR signaling module to survival regulation in monocyte-derived DC, myeloid DC and plasmacytoid DC (Figure 1).

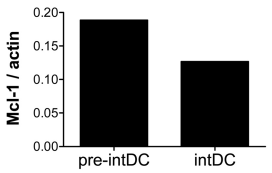

Figure 3. Mcl-1 expression decreases during differentiation of pre-interstitial DC to interstitial DC
$CD34^+$ hematopoietic progenitor cells were differentiated towards pre-interstitial DC (pre-intDC) by culture in the presence of 100 ng/ml GM-CSF, 25 ng/ml SCF, 2.5 ng/ml TNF-α and 5% heat inactivated AB^+ pooled human serum. Pre-intDC were harvested at day 7, washed thoroughly and further cultured towards interstitial DC (intDC) in the presence of 100 ng/ml GM-CSF for 7 days. Total cell lysates of pre-intDC and intDC were produced in laemmli sample buffer, protein concentrations were determined by Lowry method, equal amounts of protein were separated on SDS-PAGE and expression of Mcl-1 and actin was determined. Mcl-1 expression, normalized to actin, is shown.

Peripheral tissues contain multiple factors that adjust DC viability to situation-specific circumstances by regulating intracellular signaling. In line with this, despite their independence of GM-CSF, inhibition of NF-κB transcription factor activity induces apoptosis of terminally differentiated myeloid DC (Chapter 4), indicating that prosurvival signaling is still required (Figure 1). The altered cytokine milieu resulting from infection or tissue damage considerably affects DC survival. Furthermore, pathogen recognition, for example through TLR stimulation, directly promotes DC survival. Exposure of human monocyte-derived DC to LPS activates p38 MAPK, ERK, NF-κB and PI3K, but only PI3K appeared required to maintain LPS-induced viability (52). Another study on murine splenic DC showed that MEK1, but not NF-κB was involved in LPS-induced survival (53). In accordance, survival of LPS- and CD40 ligand (CD40L)-stimulated human monocyte-derived DC and CD34-derived Langerhans cells was relatively unaffected by addition of NF-κB inhibitors (Figure 4). CD34-derived interstitial DC could only be rescued by CD40L (Figure 4). Conversely, CpG could not rescue plasmacytoid DC treated with PI3K or PKB inhibitors (Figure 4) (54-55). These data indicate a primary role for the PI3K-PKB signaling module in modulating TLR-induced survival.

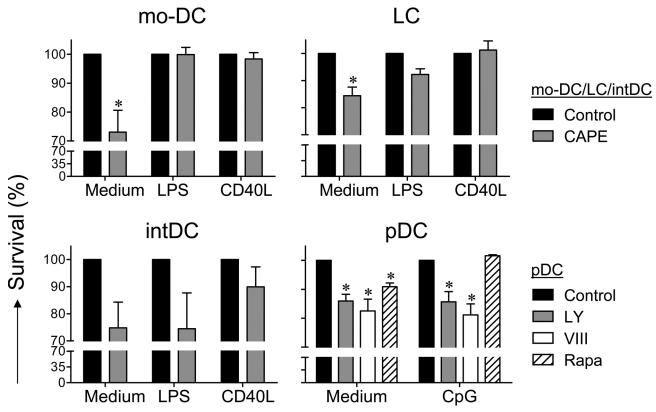

Figure 4. The role of canonical NF-κB and PI3K-PKB-mTOR activity for DC survival in the presence or absence of activation signals

*Monocyte-derived DC (mo-DC), CD34-derived Langerhans cells (LC) and CD34-derived interstitial DC (intDC) were generated in vitro. Plasmacytoid DC (pDC) were isolated from peripheral blood. DC were cultured in the presence of GM-CSF/IL-4 (mo-DC; 24 hours), GM-CSF (LC and intDC; 48 hours) or IL-3 (pDC; 24 hours), and in the presence or absence of LPS, CD40L or CpG. Canonical NF-κB inhibitor CAPE, PI3K inhibitor LY, PKB inhibitor VIII or mTOR inhibitor Rapa was added to the cultures. After the indicated culture period, apoptosis was measured by Annexin V and PI staining. AnnexinV$^-$PI$^-$ cells were considered viable. Survival was standardized to cultures without inhibitors. Mean±SEM survival is shown (mo-DC: n=4, LC and intDC: n=3, pDC: n=3). * p<0.05, paired Student t-test*

Finally, activation-induced cellular changes allow an additional level of survival regulation. CCR7 is upregulated on activated DC, and interaction with CCR7 ligands activates PKB, inhibits pro-apoptotic GSK-3β and Forkhead box O transcription factors, and induces NF-κB translocation, supporting the survival of DC migrating towards the draining lymph nodes (56). There, DC survival is further promoted by DC-T cell interaction through RANK-TRANCE and CD40-CD40L binding, resulting in activation of PKB and NF-κB (57-58). Whereas TLR-stimulation and DC-T cell interaction induce prosurvival signaling in the short term, long term effects also include the induction of pro-apoptotic factors such as Bim and NFAT (59-60), actions that appear relevant in the light of preventing uncontrolled immune responses. In conclusion, the microenvironments in peripheral tissue and secondary lymphoid organs regulate the DC lifespan to a large extent. In addition, cell-intrinsic characteristics, which may differ between distinct DC subsets, influence the consequences of specific environmental signals, thereby forming an important determinant of DC longevity.

Dendritic cell function

DC functionality has many aspects regulated through a variety of signaling modules, but the discussion below focuses mainly on the contributions of the PI3K-PKB-mTOR signaling module and canonical NF-κB transcription factors. The sentinel function of immature DC requires the continuous sampling of their antigenic environment. Murine bone marrow-derived DC (61), human monocyte-derived DC (62) as well as human CD34-derived myeloid DC (Chapter 3) generated in the presence of mTOR inhibitors show a reduced ability to take up antigen through macropinocytosis and lectin-mediated endocytosis. Thus, although after initial analysis these cells appear to have a normal DC phenotype (see above), they lack some functional abilities required for professional antigen presenting cells. In contrast, canonical NF-κB activity is essential for phenotypic DC differentiation, but cells generated in the presence of NF-κB inhibitors are equally competent at mannose receptor-mediated antigen capture as control DC (Chapter 4). The complementary roles of mTOR and NF-κB not only show that DC differentiation includes multiple components that are regulated through distinct signaling modules, but also demonstrate that it is not always possible to judge DC

differentiation based on the markers normally used for that purpose.
Whereas mTOR appears to be required to induce expression of antigen uptake receptors (62), expression of antigen presentation molecules, including MHC class II, was normal or even increased when CD34- or monocyte-derived myeloid DC were differentiated in the presence of mTOR inhibitors (Chapter 3) (7, 62). Since NF-κB is also redundant for HLA-DR expression (Chapter 4), additional signaling modules activated during myeloid DC differentiation likely mediate the induction of MHC class II molecules. Similarly, the expression of costimulatory molecules by immature myeloid DC is not dependent on PI3K-PKB-mTOR signaling or canonical NF-κB transcription factor activity (Chapters 3 and 4). More relevant than their phenotype however, are the function and immunogenicity of these DC when confronted with pathogens or danger. A variety of agents, including cortiocosteroids, vitamin D_3 and rapamycin, have been described to affect DC differentiation, yielding DC with a changed functionality (63-68). PI3K-dependent activation of mTOR (Chapter 3) (67-69) and expression of noncanonical NF-κB protein RelB (70) were shown to be required for the generation of DC that are able to properly respond to TLR ligands, inflammatory cytokines or T cell signals. Activation-induced upregulation of MHC class II and costimulatory molecules was not significantly affected in DC differentiated in the absence of sufficient mTOR or RelB activity, but these cells showed an impaired ability to secrete chemokines required to attract other immune cells. Furthermore, their reduced production of inflammatory cytokines results in an impaired ability to induce inflammation or activate T cells. Interestingly, despite the importance of canonical NF-κB in DC differentiation and inflammation in general, monocyte-derived DC differentiated under conditions of NF-κB inhibition were functionally normal (Chapter 4).
In addition to the cell-intrinsic capabilities of a DC that are established during differentiation, DC functionality is influenced by environmental signals during maturation. Canonical NF-κB is activated by many pathogen recognition receptors, including TLRs, to induce inflammatory responses in a wide variety of cell types (71). Accordingly, NF-κB is crucial for inflammatory cytokine production and appropriate T cell activation by LPS-activated myeloid DC (Chapter 4), and induces secretion of inflammatory cytokines such as IL-6 and TNF-α by plasmacytoid DC (72). Whether canonical NF-κB also regulates the expression of costimulatory molecules is less well determined. TLR-induced costimulatory molecule expression by plasmacytoid DC has been suggested to be regulated through canonical NF-κB activation (72), whereas studies on myeloid DC demonstrating NF-κB dependency and independency are both available (Chapter 4) (73-76). These differences could reflect experimental variation, but might also demonstrate subset-specific functions. Overall, activation of canonical NF-κB appears an important determinant of environment-induced regulation of DC functionality. Thereby, it makes a crucial contribution to the actions of DC in tissue as well as the immunogenicity of these DC when they arrive in the lymph nodes to interact with adaptive immune cells.
In contrast to NF-κB, the exact effect of activation-induced PI3K-PKB-mTOR signaling on DC immunogenicity is ambiguous. Intact signaling is required for CCR7-dependent lymph node migration (Chapter 3) (77). However, while clearly promoting the immunogenicity of plasmacytoid DC (Chapter 6) (78-79), the reported findings for myeloid DC are inconsistent. Actions described for PI3K and mTOR include the negative regulation of IL-12 and IL-6 and simultaneous support of IL-10 production, resulting in tolerance induction by the DC involved (6, 14, 80). In contrast, other studies have demonstrated impaired inflammatory cytokine secretion and immunogenicity following PI3K or mTOR inhibition (Chapter 3) (6, 81-82). A possible explanation for these discrepancies could lie in factors present besides DC activation stimuli, which may influence the signaling modules regulated by the activation stimulus and/or act on molecules not usually regulated by these stimuli. These

actions could change the relative contribution of specific signaling proteins to the outcome of DC activation. Since the role of the PI3K-PKB-mTOR pathway in the regulation of DC immunogenicity probably changes depending on specific circumstances, its contribution should be investigated under different conditions, preferably in vivo, to enable a reliable description of its function.

Therapeutic implications

Dysregulation of any of the determinants of DC functionality described above can result in loss of required immunity or lead to unwanted immune responses. Several pathogens have the capacity to actively block DC maturation, for example microbes like *Coxiella burnettii* and *Salmonella typhi*, and viruses such as herpes simplex virus, human immunodeficiency virus and hepatitis C virus (83). For *Escherichia coli* (84), measles virus (85-86) as well as other pathogens (85), DC inhibitory actions have been associated with inhibition of NF-κB activation. In addition to modulating maturation, pathogens can escape immunity by inducing apoptotic death, blocking lymph node migration or altering T cell stimulatory capacity of DC (83). Effects on DC differentiation have been less explored, but are likely also exploited. Not only pathogens but also tumors evade immunity by manipulating DC development, survival or function. For example, tumor-derived VEGF or IL-10 can suppress immunity by inhibiting immunogenic NF-κB and increasing tolerogenic STAT3 transcription factor activity (87-88). In contrast, inappropriate or unwanted DC activation, for example by thymic stromal lymphopoietin in allergic inflammation (89), can contribute to the development of allergy or autoimmunity, or lead to rejection of a transplanted organ.

Whereas DC may contribute to immune dysregulation due to their central role in immunity, the same capacity has resulted in them being considered in the design of novel preventive and therapeutic vaccines to counteract diseases (83, 90). In cases where classical vaccination with attenuated microbes does not elicit effective immunity, deliverance of vaccine antigens to specific receptors on DC could induce immunity, especially when accompanied by the right signals to induce DC maturation. In mice, antigen targeting to DC increased antigen presentation efficiency more than 100-fold and resulted in the development of Th1 responses against human immunodeficiency virus, Leishmania and tumors (91-93). Alternative approaches for DC-based treatment of cancer include the injection of irradiated tumor cells expressing GM-CSF to recruit DC, and injection of ex vivo generated and manipulated DC (90, 94). The latter method enables careful control of tumor antigen-loading and DC activation and has been investigated most intensively. Besides evidence on the possibility to enhance immunity using DC, application of cytokine- or otherwise modulated DC in animal models of autoimmunity or transplantation have provided proof-of-principle for the therapeutic use of tolerogenic DC (95). Although these findings provide grounds for optimism, to date, successful clinical application remains limited, particularly when DC vaccination is used in a therapeutic rather than preventive setting. For example, although tumor regression was observed in some metastatic melanoma patients vaccinated with ex vivo tumor antigen-loaded DC in phase I/II clinical trials, this was only the case in a small group of the included patients (94). Furthermore, while some clinical improvement was apparent in human immunodeficiency virus-infected patients treated with antigen-loaded monocyte-derived DC (96-97), despite considerable efforts therapeutic vaccines against other persistent viral infections such as hepatitis B virus (98-99) remain currently unavailable.

Improvement of yet ineffective DC vaccines could be achieved by better control of DC maturation and associated functional characteristics, targeting or injecting a DC subset with the right subset-specific capacites, and/or prolonging survival of well-functioning DC. Understanding

of the molecular mechanisms regulating human DC subset development, survival and function could aid in the development of protocols for the efficient ex vivo generation of sufficient numbers of optimal functioning DC. Furthermore, the identification of specific molecular targets could help in the development of novel strategies for in vivo manipulation of DC biology. The current knowledge on molecular regulation of DC development, survival and function reveals several candidates for these purposes, including signaling proteins involved in the regulation of DC numbers, the relative contribution of distinct subsets, the lifespan and the activation status of DC.

The supporting role of the PI3K-PKB-mTOR signaling module in expanding DC progenitors suggests its exploitation to boost DC yields (Chapters 3 and 6). As described in Chapter 6, yields of in vitro generated plasmacytoid DC could indeed be increased by enforced expression of a constitutively active PKB mutant, supporting the hypothesis that this axis could serve as a target to improve DC production. Based on the decisive function STAT5 has in DC lineage commitment (Chapter 5) (19, 28), it appears to be a candidate protein to manipulate DC subset-specification. Alternatively, the STAT5-regulated factors that are responsible for its effects on lineage decisions, such as PU.1 or IRF8, could be targeted. However, whereas in mice STAT5-mediated plasmacytoid DC inhibition is mediated through IRF8 (19), IRF8 loss-of-function in humans results in a more widespread DC deficiency (100), indicating that this might not be a good target in humans. RelB appears to have a subset-restricted function in both humans and mice (20, 101), and targeting RelB could provide a potential strategy to specifically regulate human interstitial DC development.

Besides manipulating subset development, the contribution of a particular subset or injected DC could be supported through regulation of its survival or activation. Enhancing PI3K-PKB-mTOR signaling promotes survival and function of human plasmacytoid DC (Chapter 6), a feature that could be used therapeutically. Mouse bone marrow-derived DC and human monocyte-derived DC expressing constitutively active PKB are highly activated DC with improved longevity that are able to eradicate tumors more efficiently than control DC (102). Similarly, siRNA-mediated deletion of the cell-intrinsic PI3K negative regulator PTEN in murine bone marrow-derived DC improved their survival, maturation, expression of CCR7 and T cell activation, resulting in increased anti-tumor immunity (103). Other potential targets more specifically involved in DC immunogenicity include STAT3 and RelB. In mice, depletion of tolerogenic STAT3 considerably enhances DC function and anti-tumor immune responses (104-105), whereas silencing of RelB results in DC inducing tolerance to transplanted hearts and treating autoimmune myasthenia gravis (106-108).

As indicated above, current attempts to manipulate DC functionality in order to enhance vaccine effectiveness have mainly focused on augmenting or attenuating the survival and/or functional abilities of existing DC. This can be achieved through injection of ex vivo generated DC as well as through in vivo targeting of specific DC subsets. At present, in vivo manipulation of the expansion or subset commitment of early progenitors does not appear feasible in humans, since human DC-specific progenitors have not yet been identified. Targeting multipotent progenitors could result in expansion of hematopoietic lineages other than DC, with unpredictable consequences. Besides the need to find a well-defined specific progenitor when wishing to manipulate development in vivo, general caution is required when aiming in vivo therapy at early progenitors due to the risk of unlimited proliferation or other unexpected side effects. Furthermore, the careful regulation of activity levels, for example required to regulate STAT5-mediated lineage choices, appears an almost impossible task in vivo. Thus, until these issues are resolved, regulation of subset specification and progenitor expansion should be applied ex vivo, where the consequences can be better controlled. Despite the remaining challenges, our knowledge on the molecular regulation of the different determinants of DC functionality contributes to the understanding of situations in

which immunological demands and responses are not well-adjusted, and will hopefully lead to novel strategies to manipulate DC biology for therapeutic purposes.

Concluding remarks

Because the induction of suitable immunogenic and tolerogenic immune responses under different situations requires correct DC subset development, survival and function, this thesis evaluated the molecular regulation of these processes for four distinct human DC subsets. Our findings have identified potential molecular targets to manipulate human DC biology for therapeutic purposes. Full application of this knowledge requires further understanding of the ontogenetic background of DC, of their relation to other antigen presenting cells, and of their contribution to the development of immune responses under different pathophysiological situations. Besides further elucidation of the molecular mechanisms regulating DC development, survival and function, future research should therefore be directed at clarification of these issues. This will hopefully lead to the use of DC with tightly controlled functional abilities in novel therapeutic applications.

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8

Summary

Dendritic cells (DC) are professional antigen presenting cells with a dual function in immunity, inducing both immunogenic and tolerogenic immune responses. The type of immune response initiated by a DC depends on its subset-specific capabilities, its lifespan determining the duration of antigen presentation, and its maturation status and associated functional characteristics. Cytokines and other factors continuously induce DC development from bone marrow-, blood- or tissue-derived progenitors and precursors. Survival and function of fully differentiated DC are dependent on their cell-intrinsic molecular signature as well as environmental stimuli. By translating environmental signals into cellular actions, signal transduction pathways provide the link between extracellular information and intracellular response, but the molecular mechanisms regulating DC biology remain poorly defined. This thesis describes intracellular signaling modules, effector proteins and transcription factors involved in the regulation of human DC subset development, survival and function.

Chapters 3 and 6 demonstrate a crucial role for the PI3K-PKB-mTOR signaling module in the development of myeloid interstitial DC and Langerhans cells as well as plasmacytoid DC, mainly through regulation of proliferation and survival of progenitor/precursor cells. Activity of canonical NF-κB transcription factors is required for the differentiation and survival of $CD34^+$ hematopoietic progenitors, CD34-derived precursor DC (pre-DC) and monocytes generating myeloid DC **(Chapter 4)**. **Chapter 5** identifies JAK2-activated STAT5 as a major regulator of CD34-derived myeloid DC differentiation. Subset-specific regulation was demonstrated by the fact that commitment to the interstitial DC but not the Langerhans cell lineage requires STAT5. High STAT5 activity in early progenitors abrogates commitment to either lineage, whereas this promotes terminal DC differentiation of committed pre-DC, showing specific requirements for the level of STAT5 activity at distinct differentiation stages.

Besides regulating survival during myeloid DC differentiation, canonical NF-κB transcription factor activity prevents apoptosis of terminally differentiated myeloid DC, although the consequences of NF-κB inhibition are less severe at later differentiation stages **(Chapter 4)**. Furthermore, the survival of fully differentiated interstitial DC and Langerhans cells is completely independent of PI3K-PKB-mTOR signaling **(Chapter 3)**. These findings could be explained by the apparently reduced need for prosurvival signaling in these cells **(Chapter 3-Appendix)**, but alternative explanations exist **(Chapter 7)**. Circulating plasmacytoid DC still require PI3K-PKB-mTOR signaling to survive **(Chapter 6)**, and inhibition of PI3K or PKB even causes apoptosis of CpG-stimulated plasmaycoid DC **(Chapter 7)**. In general however, myeloid and plasmacytoid DC viability is improved by TLR- or CD40L-induced activation **(Chapter 7)**.

Although cells with a myeloid DC-like phenotype can be generated in the absence of PI3K/mTOR activity, the resulting cells are altered with respect to antigen uptake, cytokine production and T cell stimulation **(Chapter 3)**. In contrast, inhibited canonical NF-κB transcription factor activity during monocyte-to-DC differentiation yields cells with a slightly affected phenotype, but unchanged functionality **(Chapter 4)**. Functionally improved plasmacytoid DC can be generated by increasing PI3K-PKB activity during their development **(Chapter 6)**. In addition to regulating cell-intrinsic capabilities when active during DC differentiation, the PI3K-PKB-mTOR signaling module and canonical NF-κB transcription factors have a critical function in regulating activation-induced DC functionality. Upregulation of CCR7, secretion of pro-inflammatory cytokines and the capacity to induce T cell proliferation and differentiation is promoted by activation of these signaling entities **(Chapters 3, 4 and 6)**.

In conclusion, this thesis describes the functions of three main signaling modules, PI3K-PKB-mTOR, canonical NF-κB and JAK2/STAT5, in the regulation of human interstitial DC, Langerhans cell, monocyte-derived DC and plasmacytoid DC biology. Although many details

remain to be formally proven, based on the current knowledge regarding DC progenitors and precursors **(Chapter 1)**, the cytokines and other stimuli regulating DC differentiation, maintenance and function **(Chapter 1)**, the functions of the signal transduction pathways activated by these factors and the transcription factors and other effectors involved **(Chapters 2-6)**, it is now possible to create an integrated view on the control of DC development, survival and function under different pathophysiological conditions **(Chapter 7)**. This may help understanding situations in which immunological demands and responses are not well-adjusted, for example when pathogens or tumors evade immunity or when loss of tolerance results in autoimmunity or allergy. Finally, these findings have identified potential molecular targets to manipulate DC biology for therapeutic purposes **(Chapter 7)**.

Nederlandse samenvatting

Het onderzoek beschreven in dit proefschrift gaat over het reguleren van de ontwikkeling, overleving en functie van dendritische cellen. Hieronder volgt eerst een uitleg wat dendritische cellen zijn en waarom het nuttig is de mechanismen die hun aanwezigheid en functioneren bepalen te onderzoeken. Vervolgens wordt ingegaan op de specifieke onderzoeksuitkomsten beschreven in dit proefschrift. Ten slotte wordt geschetst hoe deze bevindingen bijdragen aan de ontwikkeling van nieuwe therapieën.

Achtergrondinformatie

Dendritische cellen als regulatoren van het immuunsysteem

Een goed functionerend immuunsysteem is cruciaal voor de gezondheid. Door ziekteverwekkers zoals bacteriën en virussen aan te vallen en op te ruimen beschermt het ons tegen infectieziekten. Aan de andere kant laat het immuunsysteem ons eigen lichaam ongemoeid, wat noodzakelijk is om bijvoorbeeld auto-immuunziekten te voorkomen. Om ziekteverwekkers en lichaamseigen materiaal op tijd te identificeren en uit elkaar te houden, bevinden zich verspreid over ons lichaam een soort verkenners. Deze dendritische cellen (DC) spelen een belangrijke rol bij het aan- en uitzetten van immuunreacties.

Door verschillende signalen die de DC krijgen als ze in de buurt van bacteriën, virussen of lichaamseigen materiaal (samen de zogenaamde antigenen) zijn, herkennen ze dit als gevaarlijk of ongevaarlijk. Ze nemen deze antigenen op en kunnen deze vervolgens laten zien (presenteren) aan andere cellen van het immuunsysteem. Afhankelijk van de signalen die de DC aan deze andere immuuncellen geeft, zullen deze ofwel in actie komen tegen het gepresenteerde antigeen, ofwel het antigeen negeren en in sommige gevallen zelfs de actie van andere immuuncellen tegen dit antigeen onderdrukken. In het eerste geval ontstaat er een immuunreactie, terwijl in de twee laatste gevallen tolerantie wordt opgewekt.

Omdat DC zo bepalend zijn tijdens de beslissing tussen immuniteit en tolerantie, zijn deze cellen cruciaal voor zowel het ontwikkelen van voldoende afweer tegen ziekteverwekkers, als voor het voorkomen van ongewenste immuunreacties die kunnen leiden tot allergieën of auto-immuunziekten. Daarnaast bestaat de hoop dat met behulp van DC immuniteit tegen chronische infecties of tumoren zou kunnen worden opgewekt, tolerantie voor allergenen en lichaamseigen materiaal zou kunnen worden hersteld en zelfs tolerantie voor lichaamsvreemd materiaal zoals getransplanteerde organen zou kunnen worden geïnduceerd.

Karakteristieken die het functioneren van dendritische cellen beïnvloeden

Wanneer een DC een specifiek antigeen presenteert, kan de DC dus zowel immuniteit als tolerantie tegen dit antigeen opwekken. Het type reactie dat wordt opgewekt, is afhankelijk van een aantal karakteristieken van de DC.

- Een eerste bepalende factor is het subtype (de soort) waartoe de DC behoort. Er bestaat een grote variëteit aan DC-subtypen, met ieder hun eigen functionele mogelijkheden. De humane subtypen die aan bod komen in dit proefschrift zijn plasmacytoïde DC, interstitiële DC, Langerhanscellen en monocyt-afkomstige DC. Deze laatste drie subtypen worden samen ook wel myeloïde DC genoemd.
- Een tweede belangrijke factor is de levensduur van de DC. Dit beïnvloedt namelijk de mogelijkheden voor verkenning van de omgeving en bepaalt de duur van presentatie van het opgenomen antigeen.
- Ten slotte wordt het functioneren van een DC beïnvloed door zijn rijpings- en activatiestatus. Onrijpe (immature) DC zijn vooral goed in het opnemen en verwerken

van antigenen, terwijl rijpe (mature) DC beter zijn in antigeenpresentatie. Hun activatiestatus bepaalt vervolgens of deze mature DC een activerende of inactiverende boodschap geven aan de cellen aan wie zij antigenen presenteren. Het maturatie- en activatieniveau wordt bepaald door signalen uit de omgeving van het antigeen. Deze signalen informeren de DC over hoe gevaarlijk of ongevaarlijk het antigeen is en wat voor immuunreactie gewenst zou zijn. Hierdoor gaat de DC een op de situatie afgepaste functie uitvoeren. De functie van een specifieke DC kan dus tijdens zijn leven veranderen onder invloed van signalen uit zijn omgeving.

Regulatie van de bepalende karakteristieken door signaaltransductieroutes
De aanwezigheid van de verschillende DC-subtypen hangt af van nieuwe aanmaak van deze cellen (ontwikkeling) en van hun levensduur (overleving). De ontwikkeling en overleving worden gereguleerd door signaalstofjes, cytokinen genoemd. Ook de maturatie- en activatiestatus (functie) van een specifieke DC wordt bepaald door informatie vanuit de omgeving.
De karakteristieken van een antigeenpresenterende DC zijn dus afhankelijk van door omgevingssignalen geïnduceerde ontwikkeling, overleving en functie van DC-subtypen. De bepalende cytokinen en andere signalen worden aan de buitenkant van een DC gedetecteerd. Om vervolgens een verandering in de cel te bewerkstelligen, moet deze informatie worden vertaald. Dit gebeurt via zogenaamde signaaltransductieroutes. Deze routes kunnen worden gezien als een keten van reacties, waarbij de boodschap steeds wordt doorgegeven aan een volgende speler (de signaaleiwitten). Ten slotte leidt dit tot een actie die de cel verandert. Uiteindelijk zijn het dus de acties van signaaltransductieroutes die de ontwikkeling, overleving en functie van DC reguleren.

In dit proefschrift

Vraagstelling
Zoals hierboven uitgelegd zijn DC cruciaal voor een goed functionerend immuunsysteem. Om te begrijpen waarom het immuunsysteem in sommige situaties niet op de gewenste manier reageert en om hier eventueel op in te kunnen grijpen, is het noodzakelijk te begrijpen hoe de karakteristieken die het functioneren van DC bepalen gereguleerd worden. Deze karakteristieken zijn afhankelijk van drie processen, ontwikkeling, overleving en functie, die worden gereguleerd door signaaltransductieroutes. Het onderzoek beschreven in dit proefschrift focust zich daarom op de rol van signaaltransductieroutes in de regulatie van de ontwikkeling, overleving en functie van humane DC.

Hoofdstukindeling
Na een algemene inleiding in **hoofdstuk 1**, worden in **hoofdstuk 2** een viertal belangrijke signaaltransductieroutes beschreven: de JAK/STAT-route, de PI3K-route, de MAPK-route en de NF-κB-route. Dit hoofdstuk geeft een overzicht van de huidige kennis over de rol van deze vier routes in het reguleren van DC-ontwikkeling geïnduceerd door het cytokine GM-CSF. **Hoofdstukken 3 t/m 6** gaan in op de rol van de JAK/STAT-route, de PI3K-route en de NF-κB-route in de regulatie van de ontwikkeling, overleving en functie van humane DC-subtypen. Ten slotte worden de conclusies van de verschillende studies in dit proefschrift besproken in **hoofdstuk 7**. De belangrijkste uitkomsten worden hieronder samengevat.

Ontwikkeling
DC ontwikkelen zich vanuit hematopoietische voorlopercellen, bloedstamcellen die te

herkennen zijn aan het CD34-eiwit dat ze op hun oppervlak hebben. **Hoofdstuk 3 en hoofdstuk 6** laten zien dat voor de ontwikkeling van CD34-positieve hematopoietische voorlopercellen naar interstitiële DC, Langerhanscellen en plasmacytoïde DC, activiteit van de PI3K-PKB-mTOR-route vereist is. Cytokinen die ervoor zorgen dat CD34-positieve voorlopercellen zich ontwikkelen naar DC activeren eerst het eiwit *phosphatidylinositol 3-kinase* (PI3K). Vervolgens activeert PI3K een volgend signaaleiwit, *proteïne kinase B* (PKB), wat op zijn beurt het eiwit *mammalian target of rapamycin* (mTOR) activeert. De experimenten in **hoofdstuk 3** laten zien dat de PI3K-PKB-mTOR-route nodig is om ervoor te zorgen dat de voorlopercellen tijdens hun ontwikkeling naar myeloïde DC (interstitiële DC en Langerhanscellen) overleven. Naast overleven moeten de voorlopercellen zich vermeerderen (delen) om voldoende aantallen myeloïde DC te genereren. Ook deze celdeling blijkt afhankelijk van activiteit van de PI3K-PKB-mTOR-route. Remming van de PI3K-PKB-mTOR-route leidt daarom tot minder myeloïde DC. De cellen die ondanks deze remming ontstaan zien eruit als myeloïde DC, maar functioneren minder goed dan myeloïde DC die zich ontwikkelen onder normale omstandigheden. Dit suggereert dat PI3K-PKB-mTOR-activiteit nodig is voor de verandering (differentiatie) van stamcel naar functionele myeloïde DC. Tijdens de ontwikkeling van CD34-afkomstige myeloïde DC is de PI3K-PKB-mTOR-signaleringsroute dus verantwoordelijk voor de overleving, celdeling en in mindere mate differentie van de zich ontwikkelende cellen. Ook de ontwikkeling van plasmacytoïde DC uit CD34-positieve voorlopercellen is afhankelijk van PI3K-PKB-mTOR-signalering **(hoofdstuk 6)**. Waarschijnlijk reguleert deze route zowel celdeling, overleving als differentiatie van de voorlopercellen van plasmacytoïde DC.

Behalve PI3K, PKB en mTOR worden tijdens de ontwikkeling van myeloïde DC ook *nuclear factor-kappa B* (NF-κB)-eiwitten geactiveerd. **Hoofdstuk 4** beschrijft dat de activiteit van deze eiwitten noodzakelijk is voor de overleving van CD34-positieve voorlopercellen en van monocyten, de voorlopers van monocyt-afkomstige DC. Remming van NF-κB-activiteit verhindert bovendien de differentiatie van CD34-afkomstige interstitiële DC en monocyt-afkomstige DC.

Hoofdstuk 5 beschrijft de belangrijkste regulator van myeloïde DC differentiatie die is geïdentificeerd in dit proefschrift: *signal transducer and activator of transcription 5* (STAT5). Remming van STAT5 bevordert de differentiatie van Langerhanscellen, maar verhindert de differentiatie van interstitiële DC. Hoewel de differentiatie van interstitiële DC dus afhankelijk is van STAT5, leidt het verhogen van STAT5-activiteit in CD34-positieve voorlopercellen tot een volledig blok in hun differentiatie naar zowel Langerhanscellen als interstitiële DC. Daarentegen heeft het verhogen van STAT5-activiteit in een later ontwikkelingsstadium juist een positief effect op de differentiatie. Dit betekent dat de mate van STAT5-activiteit van cruciaal belang is voor de ontwikkeling van CD34-afkomstige Langerhanscellen en interstitiële DC en dat de gewenste hoeveelheid activiteit afhangt van het DC-subtype en het ontwikkelingsstadium.

Samenvattend spelen STAT5- en NF-κB-eiwitten een rol in de differentiatie van myeloïde DC, reguleert NF-κB bovendien de overleving van zich ontwikkelende cellen en is de PI3K-PKB-mTOR-route vooral van belang voor de overleving en celdeling van de voorlopercellen van myeloïde en plasmacytoïde DC. Mogelijkerwijs bevordert de PI3K-PKB-mTOR-route ook de differentiatie.

Overleving

De levensduur van een DC is afhankelijk van zowel celeigen kenmerken als van signalen uit de omgeving. In **hoofdstuk 6** wordt aangetoond dat PI3K-PKB-mTOR-signalering de overleving van plasmacytoïde DC uit bloed bevordert. Monocyt-afkomstige myeloïde DC hebben zowel activiteit van de PI3K-PKB-mTOR-route als van NF-κB-eiwitten nodig om

te overleven **(hoofdstuk 4)**. Net als tijdens hun ontwikkeling, is de activiteit van NF-κB-eiwitten noodzakelijk voor de overleving van volledig ontwikkelde CD34-afkomstige myeloïde DC **(hoofdstuk 4)**. Echter, de gevolgen van NF-κB-remming zijn minder ernstig in de volledig ontwikkelde cellen. **Hoofdstuk 3** beschrijft eenzelfde effect voor de PI3K-PKB-mTOR-route. Hoewel activiteit van deze route nodig is voor de overleving tijdens CD34-afkomstige myeloïde DC-differentiatie, is deze overbodig in volledig ontwikkelde myeloïde DC. Een mogelijke verklaring voor de afgenomen afhankelijkheid van deze twee signaaltransductieroutes in uitgedifferentieerde DC is de overname van overlevingsregulatie door andere signaleringsroutes, zoals wordt besproken in **hoofdstuk 7**. De experimenten in **hoofdstuk 3-appendix** suggereren echter dat volledig ontwikkelde CD34-afkomstige myeloïde DC minder afhankelijk zijn van omgevingssignalen die overleving stimuleren. Een afgenomen noodzaak voor overlevingssignalen zou ook de verminderde rol van de signaaltransductieroutes kunnen verklaren.
De signaaltranductieroutes die tijdens en na DC-ontwikkeling de overleving reguleren, kunnen dus van elkaar verschillen. Ook maturatie en activatie van DC kunnen leiden tot veranderde afhankelijkheid van specifieke signaaltransductieroutes. In het algemeen heeft DC-activatie door interactie met ziekteverwekkers een positief effect op de overleving. Dit draagt bij aan de ontwikkeling van een goede immuunreactie, omdat de DC die antigenen van deze ziekteverwekkers presenteren zo lang genoeg kunnen overleven om andere immuuncellen te activeren. De experimenten beschreven in dit proefschrift wijzen er op dat NF-κB-activatie slechts in beperkte mate bijdraagt aan activatie-geïnduceerde myeloïde DC overleving, terwijl PI3K en PKB in ieder geval bijdragen aan de activatie-geïnduceerde overleving van plasmacytoïde DC **(hoofdstuk 7)**.

Functie
DC-functie heeft vele verschillende aspecten die gereguleerd worden door een groot aantal signaaltransductieroutes, maar dit proefschrift beschrijft vooral de rol van de PI3K-PKB-mTOR-route en van NF-κB-eiwitten. Zoals hierboven beschreven kunnen CD34-afkomstige myeloïde DC zelfs ontstaan wanneer de activiteit van PI3K-PKB-mTOR wordt onderdrukt **(hoofdstuk 3)**. Echter, hun functionele capaciteiten zijn dan verminderd. De cellen kunnen minder goed antigenen opnemen en reageren minder goed op contact met een ziekteverwekker. Normaal gesproken leidt de herkenning van een ziekteverwekker of ander gevaar tot maturatie en activatie van een DC, waardoor deze goed wordt toegerust voor het activeren van het immuunsysteem. Om andere immuuncellen te kunnen activeren, communiceert een DC met deze cellen door middel van moleculen op zijn celoppervlak of door het produceren van cytokinen. DC activeren onder andere de zogenaamde T-cellen, die centraal staan bij het opwekken van een immuunreactie. De experimenten in **hoofdstuk 3** laten zien dat myeloïde DC die zijn ontstaan in de afwezigheid van PI3K-PKB-mTOR-activiteit minder cytokinen produceren dan normale myeloïde DC. Bovendien zijn zulke DC minder goed in staat om T-cellen te activeren. De PI3K-PKB-mTOR-route bevordert ook het ontstaan van goed functionerende plasmacytoïde DC **(hoofdstuk 6)**. Hoewel de activatie van NF-κB door het cytokine GM-CSF zeer waarschijnlijk een belangrijke rol speelt in de ontwikkeling van immuunactiverende DC **(hoofdstuk 2)**, lijken monocyt-afkomstige myeloïde DC die zijn ontstaan in de afwezigheid van NF-κB-activiteit normaal te functioneren **(hoofdstuk 4)**.
Door hun effecten op het ontstaan van cellen met een verbeterde of verminderde capaciteit om te reageren op ziekteverwekkers, beïnvloeden PI3K-PKB-mTOR-signalering en NF-κB-activiteit de DC-functie al tijdens zijn ontwikkeling. Daarnaast worden deze signaaltransductieroutes ook geactiveerd wanneer een DC ziekteverwekkers of andere signalen van gevaar herkent. Deze verhoging van PI3K-PKB-mTOR- en NF-κB-activiteit is nodig voor de maturatie en activatie van zowel myeloïde als plasmacytoïde DC. De

expressie van bepaalde belangrijke moleculen op het oppervlak van de DC, de productie van cytokinen en de mogelijkheid van de DC om T-cellen te activeren, worden allemaal bevorderd door de activatie van deze signaleringsroutes **(hoofdstukken 3, 4 en 6)**. De PI3K-PKB-mTOR-route en de NF-κB-route hebben dus zowel invloed op het ontstaan van functionele DC als op de functieveranderingen geassocieerd met maturatie en activatie.

Toepassingen

Nieuwe mogelijkheden voor therapie

De hierboven beschreven onderzoeksuitkomsten bieden inzicht in de regulatie van drie processen die het functioneren van DC bepalen: DC-subtype ontwikkeling, DC-overleving en DC-functie. Fouten in de regulatie van elk van deze processen kunnen leiden tot het verlies van immuniteit tegen ziekteverwekkers of van het ontstaan van immuniteit tegen lichaamseigen materiaal. Het is bekend dat verschillende bacteriën en virussen ontkomen aan detectie door het immuunsysteem door de ontwikkeling, overleving of functie van DC te beïnvloeden. Behalve ziekteverwekkers ontsnappen ook tumoren aan het immuunsysteem, terwijl ongewenste DC-activiteit bijdraagt aan de ontwikkeling van allergieën, auto-immuniteit of afstoting van een getransplanteerd orgaan. Voor een aantal van deze ziekten weten we dat ze ontstaan door manipulatie van specifieke signaaltransductieroutes. De nieuw verworven kennis over de rol van signaaltransductieroutes in de regulatie van functiebepalende karakteristieken van DC leidt tot beter inzicht in hoe ziekteverwekkers, tumoren, allergenen of donororganen 'verkeerde' gevolgen kunnen induceren. Deze kennis draagt daarmee bij aan de ontwikkeling van gerichte therapieën.

De centrale rol van DC in de beslissing tussen immuniteit en tolerantie maakt hen niet alleen tot mikpunt voor ziekteverwekkers of tumoren, maar ook tot veelbelovend 'gereedschap' voor de ontwikkeling van nieuwe behandelingen gericht op manipulatie van het immuunsysteem. Recente, vaak nog experimentele, therapieën gebruiken speciaal behandelde DC bijvoorbeeld om immuniteit tegen tumoren of virussen (bijvoorbeeld HIV) op te wekken. Daarnaast is het in ieder geval bij muizen mogelijk gebleken om met behulp van DC auto-immuniteit te behandelen en acceptatie van donororganen af te dwingen. Ondanks deze hoopvolle ontwikkelingen zijn de huidige mogelijkheden echter nog ontoereikend en zijn technieken om het functioneren van de gebruikte DC verder te beïnvloeden hard nodig. Om dit te bewerkstelligen kan gebruik gemaakt worden van de kennis over de signaaleiwitten die de ontwikkeling, overleving en functie van specifieke DC-subtypen reguleren. Zo zou bijvoorbeeld het verhogen van de activiteit van de PI3K-PKB-mTOR-route kunnen worden gebruikt om meer DC te maken, of om de bestaande DC langer te laten overleven **(hoofdstukken 3 en 6)**. Zoals beschreven in **hoofdstuk 6** is het inderdaad mogelijk om door een artificieel PKB-eiwit (myrPKB) in hematopoietische voorlopercellen te brengen, ervoor te zorgen dat er meer plasmacytoïde DC ontstaan. Daarnaast verbetert de introductie van dit myrPKB-eiwit ook de functie van deze plasmacytoïde DC, waardoor ze wellicht beter in staat zullen zijn om tumorcellen of virussen zoals HIV of hepatitis B virus aan te vallen. Hematopoietische voorlopercellen met myrPKB produceren ook meer myeloïde DC **(hoofdstuk 3)** en er bestaan al een paar voorbeelden waarin de efficiëntie van DC-therapie verbeterd is door PKB-activiteit te verhogen.

Een ander signaaleiwit dat zou kunnen worden aangepakt is STAT5, vanwege zijn cruciale rol in de beslissing welke DC-subtypen ontstaan **(hoofdstuk 5)**. NF-κB **(hoofdstuk 4)** en mTOR **(hoofdstuk 3 en hoofdstuk 6)**, maar ook andere signaaleiwitten, spelen een belangrijke rol bij de maturatie en activatie van DC. De manipulatie van deze eiwitten kan leiden tot grote veranderingen in de immuunreacties aangezet door DC. In muizen is dit al

toegepast om verbeterde immuniteit tegen tumoren te bewerkstelligen, om tolerantie voor transplantaten te induceren en om auto-immuunziekten te behandelen.

Conclusie

De conclusies van het onderzoek beschreven in dit proefschrift dragen bij aan de huidige kennis over de rol van signaaltransductieroutes in de regulatie van de ontwikkeling, overleving en functie van DC-subtypen. Deze kennis heeft verschillende aanknopingspunten voor nieuwe behandelmethoden gebaseerd op DC opgeleverd. Het draagt bij aan het begrip van situaties waarin immuunreacties niet goed zijn afgestemd op de omstandigheden en zal hopelijk leiden tot het ontwikkelen van nieuwe strategieën om DC te manipuleren voor gebruik in immuuntherapie.

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PhD portfolio

Name PhD student	Lianne van de Laar
Erasmus MC department	Gastroenterology and Hepatology
PhD period	2007 – 2011
PhD advisors	Prof.dr. P.J. Coffer (UMC Utrecht) Prof.dr. H.L.A. Janssen Dr. A.M. Woltman

Summary of PhD training activities

General academic and research skills: courses

2009 Advanced Course on Immunology, Erasmus Postgraduate School Molecular Medicine, Erasmus University, Rotterdam

2010 Guiding the actions of the immune system, Dutch Society for Immunology, Lunteren

2010 Writing Successful Grant Proposals, Erasmus Postgraduate School Molecular Medicine, Erasmus University, Rotterdam

2011 Time for high T, Dutch Society for Immunology, Lunteren

International conferences: oral and poster presentations

2008 American Association for the Study of Liver Disease, San Francisco, CA, USA
Intact PI3K-PKB-mTOR signaling is crucial for human myeloid dendritic cell development, but not maturation. Poster

2008 European Macrophage and Dendritic Cell Society, Brescia, Italy
CD34-derived myeloid dendritic cell development requires intact PI3K-PKB-mTOR signaling. Poster

2009 Keystone Symposium Dendritic cells/Toll like receptors, Banff, Canada
CD34-derived myeloid dendritic cell development requires intact PI3K-PKB-mTOR signaling. Poster

2009 European Conference on Immunology, Berlin, Germany
CD34-derived myeloid dendritic cell development requires intact PI3K-PKB-mTOR signaling. Oral

2010 DC2010: Forum on Vaccine Science, Lugano, Switzerland
Ectopic protein kinase B augments human CD34-derived plasmacytoid dendritic cell development; a possible target for immunotherapy? Poster

2011 3rd International Workshop on Plasmacytoid Dendritic Cells and Immune Responses, Paris, France
The PI3K-PKB-mTOR-S6K axis is a potential therapeutic target to stimulate dysfunctional plasmacytoid dendritic cells of chronic hepatitis B virus-infected patients. Oral

National conferences: oral and poster presentations

2006 Annual Conference Dutch Society for Immunology, Noordwijkerhout
CD34-derived myeloid dendritic cell development requires intact canonical NF-κB signaling. Oral

2007 Annual Conference Dutch Society for Immunology, Noordwijkerhout
CD34-derived myeloid dendritic cell development requires intact PI3K-PKB-mTOR signaling. Poster

2008 Annual Conference Dutch Society for Hepatology, Veldhoven
CD34-derived myeloid dendritic cell development requires intact PI3K-PKB-mTOR signaling. Poster
2008 Annual Conference Dutch Society for Immunology, Noordwijkerhout
CD34-derived myeloid dendritic cell development requires intact PI3K-PKB-mTOR signaling. Oral
2009 Annual Conference Dutch Society for Immunology, Noordwijkerhout
The role of canonical NF-κB in human myeloid dendritic cell development and function. Poster
2009 Annual Conference Dutch Society for Immunology, Noordwijkerhout
Ectopic protein kinase B augments human CD34-derived plasmacytoid dendritic cell development; a possible target for immunotherapy? Oral
2009 Molecular Medicine Day, Erasmus University, Rotterdam
CD34-derived myeloid dendritic cell development requires intact PI3K-PKB-mTOR signaling. Oral
2009 Annual Conference Dutch Society for Hematology, Papendal
CD34-derived myeloid dendritic cell development requires intact PI3K-PKB-mTOR signaling. Oral
2010 Annual Conference Dutch Society for Immunology, Noordwijkerhout
Tight control of STAT5 activity determines human CD34-derived interstitial dendritic cell and Langerhans cell development. Oral
2010 Molecular Medicine Day, Erasmus University, Rotterdam
Ectopic protein kinase B augments human CD34-derived plasmacytoid dendritic cell development; a possible target for immunotherapy? Poster
2011 Annual Conference Dutch Society for Hepatology, Veldhoven
Ectopic protein kinase B augments human CD34-derived plasmacytoid dendritic cell development; a possible target for immunotherapy? Poster
2011 Annual Conference Dutch Society for Immunology, Noordwijkerhout
The PI3K-PKB-mTOR-S6K axis is a potential therapeutic target to stimulate dysfunctional plasmacytoid dendritic cells of chronic hepatitis B virus-infected patients. Oral

National conferences: attendance
2007 DLA Science Day, UMC Utrecht, Utrecht
2007 Mini-symposium: Biology of hematopoietic stem cells, Erasmus MC-University Medical Center Rotterdam, Rotterdam
2007 Erasmus Liver Day, Erasmus MC-University Medical Center Rotterdam, Rotterdam
2008 Annual Conference Dutch Society for Hematology, Papendal
2008 Erasmus Liver Day, Erasmus MC-University Medical Center Rotterdam, Rotterdam
2010 Mini-symposium: Virus-Host Interactions and the Regulation of Viral Immunity, Erasmus MC-University Medical Center Rotterdam, Rotterdam

Teaching activities: supervision internships BSc and MSc students
2008 A. van den Bosch
2009 H. Hidare
2010 E. van der Aa
2011 S. Gordijn

Scientific awards and grants
2008 Travel grant; Dutch Society for Immunology

2008 Travel grant; Trustfonds Erasmus University
2009 2nd prize winner for the best oral presentation; Molecular Medicine Day, Erasmus University
2010 Travel grant; Trustfonds Erasmus University
2011 Funded visit to Feinberg graduate school; Weizmann Institute of Science, Israel

Curriculum vitae

De auteur van dit proefschrift werd geboren op 29 juli 1983 te Terheijden. In 2001 behaalde zij haar Gymnasiumdiploma *cum laude* aan het Onze Lieve Vrouwelyceum te Breda. Datzelfde jaar startte zij met haar studie Biomedische Wetenschappen aan de Universiteit Leiden. Het vierde semester van haar studie nam zij deel aan een intracurriculaire uitwisseling met de opleiding Biomedicine aan het Karolinska Instituut te Stockholm, Zweden. In het laatste jaar van haar Bachelor liep zij stage op de afdeling Nierziekten (Dr. A.M. Woltman) van het Leids Universitair Medisch Centrum (LUMC). Een beurs vanuit het 'Excellente Studenten Traject' maakte het mogelijk dit onderzoek naar functionele modulatie van dendritische cellen na afloop van de stage naast haar studie parttime voort te zetten. Het Bachelorexamen werd in 2004 *cum laude* behaald. De eerste stage in de Masterfase werd gevolgd bij de afdeling Diabetes Research Laboratories, Oxford Center for Diabetes, Endocrinology and Metabolism, University of Oxford, UK (Dr. A. Clark en Dr. E.J.P. de Koning). Dit onderzoek naar de rol van amyloïd en bètacelneogenese in de pathologie van type 2 diabetes mellitus werd beloond met de LUMC Student Research Award 2005. Vervolgens onderzocht zij de rol van Rev7 in DNA synthese tijdens een extra stage bij de afdeling Toxicogenetica van het LUMC (Dr. G. Hendriks). Voor haar afstudeerstage keerde zij terug naar de afdeling Nierziekten (Prof.dr. C. van Kooten en Dr. A.M. Woltman), waar zij onderzoek deed naar de rol van NF-κB tijdens het ontstaan van dendritische cellen. In januari 2007 behaalde zij haar Masterexamen *cum laude*. In februari startte zij met het in dit proefschrift beschreven promotieonderzoek aan de afdeling Maag- Darm- en Leverziekten van het Erasmus MC-Universitair Medisch Centrum Rotterdam, onder begeleiding van Dr. A.M. Woltman, Prof. dr. P.J. Coffer en Prof.dr. H.L.A. Janssen. Een gedeelte van dit onderzoek werd verricht aan de afdeling Immunologie, Universitair Medisch Centrum Utrecht, waar zij een jaar werkte in het Molecular Immunology Lab geleid door Prof.dr. P.J. Coffer. Sinds februari 2011 is zij aangesteld als post-doc bij de afdeling Maag- Darm- en Leverziekten van het Erasmus MC-Universitair Medisch Centrum Rotterdam, waar zij haar onderzoek naar de regulatie van het ontstaan en functioneren van humane dendritische cellen gedurende een jaar zal voortzetten.

List of publications

van de Laar, L., A. van den Bosch, A. Boonstra, R.S. Binda, M. Buitenhuis, H.L.A. Janssen, P.J. Coffer, and A.M. Woltman. Enhanced PI3K-PKB activity augments human plasmacytoid dendritic cell development and function. *Manuscript in preparation*

van de Laar, L., P.J. Coffer, and A.M. Woltman. Regulation of dendritic cell development by GM-CSF: molecular control and implications for immune homeostasis and therapy. *Submitted*

van de Laar, L., A. van den Bosch, A.T.J. Wierenga, H.L.A. Janssen, P.J. Coffer, and A.M. Woltman. 2011. Tight control of STAT5 activity determines human CD34-derived interstitial dendritic cell and Langerhans cell development. *J. Immunol.* 186:7016-7024

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van de Laar, L., M. Buitenhuis, F.M. Wensveen, H.L.A. Janssen, P.J. Coffer, and A.M. Woltman. 2010. Human CD34-derived myeloid dendritic cell development requires intact phosphatidylinositol 3-kinase-protein kinase B-mammalian target of rapamycin signaling. *J. Immunol.* 184:6600-6611.

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Clark, A., J. Moffit, L. van de Laar, K. Pinnick, F. Sayyed. 2008. Pancreatic Islet Pathophysiology and Pathology in Obesity. In *Metabolic Syndrome: Epidemiology, Clinical Treatment, and Underlying Mechanisms*, B. C. Hansen, G. A. Bray, ed. Humana Press, New Jersey, USA