Transcriptional control of human plasmacytoid dendritic cell and B cell differentiation
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Regulation of human plasmacytoid dendritic cell maturation and function by Spi-B
Regulation of human plasmacytoid dendritic cell maturation and function by Spi-B

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Abstract

Plasmacytoid dendritic cells (pDCs) act as professional, i.e. highly specialized effector cells at two different stages of differentiation: immature pDCs produce high levels of type I IFNs upon stimulation by viruses or bacteria, whereas upon maturation, pDC-derived DCs present antigen, induce T cell priming and lose the capacity to produce IFN. It is not known how the cellular machinery enables the dramatic morphological and functional changes accompanying this transition. Here we investigated the role of the Ets factor Spi-B which, amongst cells of the DC lineage, is exclusively expressed in pDCs. Spi-B protein was strongly decreased upon stimulation of ex vivo isolated pDCs. Ectopic expression of Spi-B by retroviral transduction in pDCs generated from human progenitors resulted in increased maturation: Spi-B overexpressing pDCs were hyperactivated and expressed higher levels of co-stimulatory and MHC class II molecules on the surface, before, as well as after stimulation. In contrast, decreased Spi-B levels by short hairpin (sh) RNA in a pDC cell line abrogated CD40, CD80 and CCR7 upregulation upon stimulation. Furthermore, high Spi-B levels inhibited production of IFN-α and the pro-inflammatory cytokines TNF-α and IL-6 upon TLR ligation. We conclude that Spi-B is involved in regulating the differentiation of pDCs, which upon stimulation can secrete cytokines and prime T cells.
Introduction

During viral infection, plasmacytoid dendritic cells (pDCs) are crucially involved in linking innate and adaptive immunity. By secreting large amounts of type I interferons (IFNs) and other cytokines and by cell-contact dependent mechanisms as antigen presentation, pDCs modulate the strength, duration and quality of cDC, NK, B and T cell responses. The transition of naïve pDCs into mature antigen presenting or cytokine producing pDCs is accompanied by major morphological, structural and functional changes.

pDCs selectively express Toll-like receptor (TLR)7 and TLR9, but are devoid of expression of other TLRs which are otherwise found in cDCs. pDCs are therefore specialized in sensing microbial and viral nucleic acid: TLR7 ligands include viral ssRNA as from HIV or influenza virus, whereas unmethylated CpG oligonucleotides, which are abundant in bacterial DNA, potently activate TLR9. After being taken up through an endocytic pathway, CpG DNA or viral ssRNA bind to endosome-anchored TLR9 or TLR7; this binding triggers the recruitment of the myeloid differentiation primary response gene 88 (MyD88) adaptor molecule. MyD88 complexes with members of the IL-1 receptor-associated kinase (IRAK) family, IRAK1 and IRAK4, and they further associate with interferon regulatory factor (IRF) transcription factors IRF7 and IRF5, tumor necrosis factor receptor-associated factors TRAF6 and TRAF3, and IkB kinase-α (IKKα). After being phosphorylated by IRAK1 and activated by TRAF6, IRF7 translocates into the nucleus to initiate type I IFN transcription, and thus serves as a master mediator for IFN responses. IRF5 nuclear translocation, together with NF-κB and MAPK activation, is crucial for the production of inflammatory cytokines.

Immature pDCs express low levels of MHC class II and low to undetectable levels of co-stimulatory molecules (CD40, CD80, CD86) and are therefore incapable of inducing significant antigen-specific T cell proliferation (reviewed in). Stimulation of pDCs results in their maturation by upregulation of MHC class I and II as well as co-stimulatory molecules. In vitro studies suggest that pDCs can differentiate into mature DCs by two distinct pathways a) by triggering IL-3R, which is highly expressed on pDCs, in cultures with IL-3 or IL-3 and CD40L or b) by triggering TLR7/9 by viruses or by synthetic CpG ODN. pDCs matured by IL-3 and CD40L preferentially induce Th2 priming, whereas pDCs matured by virus can prime T cells to produce IFN-γ and IL-10. As TLR triggering on pDCs also leads to production of IFN-α, as well as TNF-α, IL-6 and several chemokines it can be stated that pDCs act as professional effector cells at two different levels. As cytokine producing cells and as T cell priming antigen-presenting cells. It is currently unclear if these are consecutive differentiation stages or alternative fates of activated pDCs and how the differentiation of pDCs is established by molecular regulation. Understanding this process could have important clinical implications, considering the link between a dysregulated TLR-induced IFN response and autoimmune diseases.

Here we investigated the role of the Ets factor Spi-B in pDC maturation. We previously reported that Spi-B is highly expressed in pDCs and absent from other DC subtypes. Ectopic expression of Spi-B in human hematopoietic progenitors inhibits their development into T, B or NK cells, but supports their development into pDCs. Spi-B is required for the development of pDCs from human progenitors, as this is strongly prevented by decreasing Spi-B levels by shRNA.

Spi-B protein, unlike Spi-B mRNA, was strongly decreased upon stimulation of ex vivo isolated pDCs by TLR, CD40 and/or IL-3 receptor triggering. Ectopic expression of Spi-B by retroviral transduction in pDCs generated from human progenitors inhibits their development into T, B or NK cells, but supports their development into pDCs. Spi-B expression prevents the optimal production of IFN-α and the inflammatory cytokines TNF-α and IL-6 upon stimulation of pDCs. We therefore conclude that Spi-B, next to its crucial requirement for development, is involved in the functional differentiation of pDCs. High Spi-B levels promote the antigen-presenting pDC fate while preventing cytokine production by pDCs.
Materials and methods

Isolation CD34⁺CD1a⁻ progenitors, pDCs and cDCs from postnatal thymus and peripheral blood. Buffy coats for isolation of pDCs and cDCs were obtained from Sanquin Bloodbank, Amsterdam, The Netherlands. Human thymocytes were obtained from postnatal thymus as described before 17. Peripheral blood and thymic cell suspensions were separated by ficoll gradient and CD34⁺, BDCA4⁺ and CD11c⁺ cells were isolated by MACS cell separation. Highly pure populations of CD34⁺CD1a⁻ progenitors, Lin⁻CD123⁺CD45RA⁺ pDCs and Lin⁻HLA-DR⁺CD11c⁺ cDCs were obtained by FACS cell sorting.

OP9 cultures. pDCs and cDCs were generated in vitro from CD34⁺CD1a⁻ hematopoietic precursors isolated from PNT in OP9 co-cultures as described before 17. Briefly, CD34⁺CD1a⁻ progenitors were cultured on a layer of OP9 stromal cells in the presence of 5ng/ml Flt3L and 5ng/ml IL-7. pDCs were analyzed after 7 days of co-culture. In vitro generated DCs, CD123⁺BDCA2⁺HLA-DR⁻ pDCs and HLA-DR⁺CD123⁺ cDCs were isolated after 10 days of co-culture by FACS sorting.

Flow cytometry. Monoclonal antibodies against human CD80, CD86, HLA-DR, CD123, CD45RA directly conjugated with FITC, PE, PE-Cy7 or APC-Cy7 were purchased from BD PharMingen, CD40-PE from Immunotech, BDCA2-APC from Miltenyi Biotec. Samples were analyzed by flow cytometry on a LSRII (BD) and analyzed using FlowJo software (TreeStar).

Cell lines. The OP9 murine bone marrow stromal cell line was used as described before 17. Cells were maintained in culture with MEMα medium (Invitrogen, Carlsbad, CA) with 20% FCS (Hyclone, Logan, UT). CAL-1 cells were kindly provided by T. Maeda 18, Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, and cultured in RPMI supplied with 8% FCS.

Constructs and retroviral transductions. The retroviral constructs LZRS-IRES-GFP and pTRIP-GFP used to overexpress or knock down Spi-B were described previously 15,16. Retroviral transductions of hematopoietic stem cells were performed as described before 17.

Immunoblot analysis. Western blotting was performed as described 15. Briefly, whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane. After blocking with TBST containing 5% milk, membranes were incubated with antibodies against human Spi-B 19 (kindly provided by Lee Ann Sinha, State University of New York, Buffalo, USA) or Actin (I-19, Santa Cruz Biotechnology). Bands were visualized by using secondary horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO) and chemiluminescent substrate (Pierce).

Quantitative PCR. Total mRNA was isolated from cells using RNeasy mini kit (Qiagen) and reverse transcribed into cDNA with first strand buffer, superscript II reverse transcriptase (Invitrogen), dNTP (Roche) and Oligo(dT) (Promega). Primer sequences for actin 20, Spi-B 17 and CD40 21 are as published.

Cytokine detection by ELISA. INF-α concentrations were assessed with an IFN-α ELISA kit according the manufacturers protocol (Biosource International). Measurements of IL-6 and TNF-α was performed by ELISA using pairs of specific mAbs and recombinant standards. IL-6 mAbs were obtained from Biosource International, rIL-6 was from R&D. TNF-α mAbs and standard were purchased from eBioscience. Biotinylated antibodies were detected with Strept-HRP obtained from Sanquin.
Chapter 3

Results

**Spi-B protein is degraded upon stimulation of pDCs.**

We have shown previously that Spi-B is expressed at the mRNA and protein level in pDCs isolated from human tonsils but not in monocyte-derived DCs (Mo-DC)\(^1\). Consistently, the expression of Spi-B is required for human pDC, but not for cDC development\(^2\). To extend the finding that Spi-B expression is restricted to pDCs within the various DC subsets, we analyzed Spi-B expression by real-time PCR in pDCs and cDCs isolated from peripheral blood (PB) or post-natal thymus (PNT), as well as pDCs and cDCs generated in vitro from CD34\(^+\)CD1a\(^-\) progenitors isolated from PNT in the presence of Flt3L cytokine\(^2\) (Figure S1). Our results show that Spi-B mRNA levels are more than 100-fold higher in pDCs isolated from PB or PNT compared to CD11c\(^+\) cDCs, and that in vitro generated pDCs express approximately 30-fold higher Spi-B levels as their conventional in vitro generated counterparts.

pDCs express IL-3 receptor (CD123), CD40 and TLR7/9 and can therefore be activated with IL-3, IL-3 and CD40L, and also with TLR-ligands such as CpG, virus or imiquimod. We previously reported that Spi-B mRNA levels are unchanged in pDCs after stimulation with IL-3 or IL-3 plus CD40L for 12 to 48 hours\(^1\). In order to investigate the effect of TLR stimulation on Spi-B expression in pDCs, pDCs isolated from PNT were cultured for 5h, 24h or 48h in the presence of CpG 2216 (CpG-A) or CpG 2006 (CpG-B), imiquimod, IL-3, IL-3 and CD40L or without stimulus as control. While activation of pDCs induced upregulation of surface expression of CD40 and CD86 (Supplemental figure 2), as described previously by Cella et al.\(^8\), Spi-B expression by quantitative PCR revealed that none of the stimuli tested affected Spi-B mRNA levels after 5h or 24h of culture (data not shown). As our previous studies indicated that Spi-B may be post-translationally regulated, we assessed protein levels of Spi-B before and after activation of pDCs. Same numbers of pDCs were either collected directly after sorting or cultured 5h, 24h or 48h in the presence of CpG, imiquimod, or IL-3 and CD40L (Figure 1). Stimulation for 5h did not affect Spi-B protein levels. On the contrary, Spi-B protein was strongly diminished in pDCs stimulated for 24h or 48h, respectively. The decrease in Spi-B protein was independent of the mode of stimulation. Culturing in medium without stimulation led to massive cell death, as confirmed by FACS analysis (data not shown) and resulted in low levels of actin (the loading control) hampering comparison of protein levels between freshly isolated and pDCs cultured 24h in medium only. Addition of IFN-\(\alpha\), which is a survival factor but does not activate pDCs\(^2\) to the culture medium largely prevented cell death and decrease in actin protein. Spi-B protein levels in pDCs treated with IFN-\(\alpha\) also decreased to almost undetectable levels after 24h or 48h of culture.

![Figure 1. Spi-B protein is degraded upon stimulation of pDCs.](image1)

CD123\(^+\)CD45RA\(^+\) pDCs were isolated from postnatal thymus and equal numbers of cells were collected directly or stimulated with various stimuli for 5h, 24h or 48h as indicated. Cell lysates were analyzed by immunoblotting for Spi-B and actin protein. Lysates from CAL-1 cells transduced with LZRS Spi-B served as positive control for Spi-B protein detection. One representative experiment out of three is depicted.
Collectively we confirmed that Spi-B expression is restricted to pDCs within DC subpopulations and conclude that activation of pDCs, independent of the signaling pathway, leads to a strong decrease of Spi-B protein levels, which is not due to reduction of Spi-B transcription but possibly to post-translational regulation of Spi-B.

**Ectopic Spi-B expression results in a hyperactivated pDC phenotype.**

We previously described that ectopic expression of Spi-B by retroviral transduction in CD34⁺CD1a⁻ progenitors promotes pDC development, leading to the generation of higher numbers of pDCs in Flt3L containing cultures compared to controls. To assess how Spi-B overexpression affects the pDC phenotype, we transduced progenitors with the Spi-B overexpression construct and cultured the cells in Flt3L containing cultures. GFP⁺CD123hi pDCs were phenotypically analyzed at 7 days after transduction. Consistent with our previous findings, ectopic Spi-B expression increased the percentage of BDCA2⁺CD123hi pDCs 2-10 fold (data not shown). Increasing Spi-B levels resulted in significantly increased surface expression of CD40, the co-stimulatory molecule CD80 and MHC class II (HLA-DR) (Figure 2 and 3). Higher levels of CD86 were measured on Spi-B transduced pDCs, but the increase was not statistically significant. Thus, Spi-B protein levels affect the phenotypic maturation state of pDCs.

**Enhanced upregulation of co-stimulatory and MHC molecules by Spi-B after stimulation.**

As Spi-B protein is degraded upon stimulation we were interested to assess the effect of increased Spi-B levels on the maturation of pDCs upon activation. Similar to ex vivo isolated pDCs, activation of in vitro generated pDCs by CpG or IL-3R/CD40 triggering lead to an increase in surface level expressions of maturation markers (Figures 3 and S3).

Upon stimulation of pDCs with CpG 2216 or CD40L/IL-3, the mean fluorescence intensity (MFI) of CD40 surface expression increased 15-fold, of CD80 10-fold, of CD86 4- to 5-fold and of MHC class II (HLA-DR) 1.5- to 2-fold compared to unstimulated pDCs. Ectopic Spi-B expression steadily and significantly further elevated expression levels of these maturation markers (Figure 3). CD40 MFI was further increased by ectopic Spi-B by 2-fold, CD80 and CD86 MFI by >1.5-fold and HLA-DR by >1.3-fold.

In conclusion, the surface expression of markers characteristic for a mature pDC phenotype are induced by overexpression of Spi-B. This Spi-B-mediated hyperactivated pDC phenotype is retained after stimulation of pDCs by TLR or CD40/IL-3R triggering.

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**Figure 2. Ectopic Spi-B expression induces a more mature pDC phenotype.** CD34⁺CD1a⁻ progenitors from PNT were transduced with Spi-B LZRS GFP or control LZRS GFP and cultured on OP9 cells for 7 days. Surface expression of CD40, CD80, CD86 and MHC class II on GFP⁺CD123hi cells transduced with control virus (solid grey histograms) or Spi-B virus (black line histograms) are depicted. One representative experiment out of seven is shown.
Figure 3. Upregulation of co-stimulatory and MHC molecules after stimulation is enhanced by ectopic Spi-B. CD34+CD1a- progenitors isolated from PNT were with LZRS Spi-B GFP or control GFP vector and cultured on OP9 cells for 7 days. Bulk cultures were stimulated overnight with the stimuli indicated. Expression of CD40, CD80, CD86 and MHC class II on GFP^CD123^hi cells was measured and mean fluorescence intensities (MFI) relative to unstimulated control transduced cells were calculated. Average relative MFIs of seven independent experiments are depicted. Student’s t-test, * p < 0.05, ** p < 0.01, ns: not significant.

**Spi-B is required for the induction of maturation markers on pDCs.**

In order to assess whether Spi-B is crucial for upregulation of co-stimulatory and MHC class II molecules after stimulation, we investigated reduction of Spi-B levels in pDCs on their maturation stage. Since pDCs are non-cycling cells retroviral transduction of mature ex vivo isolated pDCs is not possible. Therefore, to answer this question, we made use of the pDC cell line CAL-1, which was established from CD4^+^CD56^+^ hematodermic neoplasm cells of a 76-year old male patient. The pDC origin of this tumor has previously been confirmed. Upon overnight stimulation with CpG 2006 CAL-1 cells strongly upregulate the co-stimulatory molecules CD40 and CD80 as well as the chemokine receptor CCR7 on their surface (Figure 4). Unlike their primary counterparts, CAL-1 cells express high baseline surface levels of CD86 and MHC class II that are only weakly increased upon stimulation (and data not shown); therefore these maturation markers were not assessed in this experiment. Like in primary cells, overexpression of Spi-B in CAL-1 cells increased surface expression of CD40 and CD80 compared to control transduced cells, which was persistent upon stimulation of the cells (Figure 4A). To reduce Spi-B protein levels we made use of a retroviral shRNA construct described previously, and observed efficient decreases in Spi-B protein levels in CAL-1 upon transduction (Figure S4). Decreased Spi-B levels in CAL-1 prevented TLR9-mediated upregulation of CD40, CD80 and CCR7 (Figure 4A). For example, activation of control-transduced cells increased CD40 levels approx. 30-fold after transduction, activation of CAL-1 cells transduced with Spi-B shRNA only increased CD40 levels approx. 4-fold. This data indicates a role of Spi-B in TLR9-mediated upregulation of maturation markers on pDCs. The impact of Spi-B on CD40 surface protein levels was due to regulation of CD40 gene expression by Spi-B (Figure 4B) since Spi-B overexpression in CAL-1 cells resulted in approx 30-fold increase in CD40 transcription in unstimulated cells compared to controls. Moreover CD40 gene expression, which was strongly induced by TLR9 triggering, was still 10-fold higher after stimulation in Spi-B overexpressing cells as in control transduced cells (Figure 4B). Induction of CD40 gene expression upon TLR9 triggering was 2-fold lower in CAL-1 cells transduced in which Spi-B levels were decreased by shRNA. We and others previously described that CD40 gene expression is directly regulated by Spi-B in macrophages and B cells. Most likely, Spi-B also directly regulates CD40 expression in pDCs. From this observation we conclude that Spi-B is required for the activation-induced upregulation of CD40 and possibly other maturation surface molecules such as CD80 and CCR7 upon stimulation of pDCs.
**Spi-B in pDC maturation and function**

**Figure 4. Spi-B is required for the induction of phenotypic maturation upon stimulation.** CAL-1 cells were transduced with Spi-B LZRS GFP (Spi-B), Spi-B shRNA GFP (SpiB-i) or control constructs (ctrl, Renilla-i) and stimulated with CpG 2006 at day 6 after transduction. (A) CD40, CD80 and CCR7 surface expression on transduced GFP+ cells are shown. (B) Transduced cell were sorted and CD40 gene expression was analyzed by quantitative PCR. Values are normalized to expression in control transduced, unstimulated cells. Averages ± SD of PCR duplicates are shown. One representative experiment out of three is displayed. RCN, relative cell numbers.

**Ectopic Spi-B expression reduces cytokine production after pDC stimulation.**

The main cytokine produced by pDCs is type I IFN, which is secreted upon simulation of TLR7 or TLR9. Moreover, human pDCs are able to produce IL-6 and TNF-α but not IL-12, upon TLR7 or TLR9 triggering\(^7,26\). As pDCs mature, they upregulate MHC and co-stimulatory molecules, which enables antigen presentation, while losing their ability to produce IFN-α\(^8,27\). Given the role of Spi-B in activation of pDCs we investigated the effects of Spi-B overexpression in pDCs to produce and secrete cytokines after stimulation. For this purpose, Spi-B and control transduced GFP\(^+\)CD123\(^hi\) pDCs were sorted from OP9 cultures on day 6 and stimulated overnight with TLR9 ligand CpG 2216, and IFN-α, IL-6 and TNF-α concentrations were measured in the supernatants by ELISA. Spi-B transduced pDCs produced 2- to 3-fold less IFN-α than control pDCs (Figure 5). Also IL-6 and TNF-α production was strongly impaired in Spi-B overexpressing pDCs. Cytokine concentrations in supernatant of unstimulated cells were below detection levels. This data suggests that high Spi-B levels prevent secretion of IFN-α, IL-6 and TNF-α upon stimulation.
Figure 5. Spi-B overexpression prevents IFN-α and pro-inflammatory cytokine production of pDCs after stimulation. CD34+CD1a- progenitors isolated from PNT were transduced with Spi-B LZRS GFP or control virus and cultured on OP9 cells for 6 days. GFP⁺CD123⁺ pDCs were sorted and stimulated with CpG 2216 for 24h. Concentrations of the indicated cytokines in the supernatant were assessed by ELISA. One representative experiment out of three is shown. nd, not detected.
Discussion

The data presented in this report indicate that the Ets factor Spi-B plays a role in the functional differentiation of pDCs (Figure 6). Among DCs, Spi-B transcription was restricted to the plasmacytoid subset, as investigated in DCs from peripheral blood, thymus and generated from hematopoietic progenitors in Flt3L cultures. Overexpression of Spi-B levels enhanced the expression levels of co-stimulatory and MHC class II molecules on immature but also on stimulated pDCs relative to controls. Concomitantly, high Spi-B levels decreased secretion of IFN-α and inflammatory cytokines upon TLR9 triggering. Knock-down of Spi-B protein affected the surface expression levels of maturation markers on the pDC cell line CAL-1 and reduced their upregulation upon stimulation.

Supposedly, the previously reported effect of Spi-B on pDC development and the here described effect of Spi-B on pDC maturation and function may be accounted to potential survival properties of Spi-B in pDCs, i.e. high Spi-B levels may prolong the survival of pDCs in bulk progenitor Flt3L cultures, leading to increased pDC percentages and a hyperactivated phenotype. To assess the effect of increased survival of pDCs on their phenotype we transduced CD34+ progenitors with the well known anti-apoptotic factors Bcl-2 and Bcl-xL (reviewed by Reed28) and investigated their effect in Flt3L pDC cultures (data not shown). Overexpression of Bcl-2 and Bcl-xL lead to increased absolute cell numbers within the transduced population of the bulk culture, confirming the functionality of the constructs, but ectopic expression of Bcl-2 of Bcl-xL, unlike Spi-B transduction, did not specifically increase pDC numbers. Also, Bcl-2 or Bcl-xL did not affect CD40, CD80, CD86 or HLA-DR expression levels on pDCs before and after stimulation as compared to control transduced cells. This data shows that that increased cell survival by ectopic expression of anti-apoptotic proteins does not account for the observed effects of Spi-B on pDC development, maturation and function.

Given the significance of type I IFNs in activating a wide range of innate and adaptive immune cells and the involvement of IFN-α in several autoimmune diseases, it is clear that IFN-α secretion needs be under tight control. Multiple surface receptors have been described to be involved in regulating the TLR-mediated responses in pDCs: Siglec-H and NKP44, P2Y receptors, C-type lectins BDCA2 and DCIR as well as the pDC-specific receptor ILT7 suppress the ability of pDCs to secrete IFNs upon TLR ligation29-36. Next to reducing type I IFN secretion cross-linking of BDCA2 or ILT7 also regulates secretion of the pro-inflammatory cytokines IL-6 and TNF-α29,30. This is interesting, as the transcription of the IFN-α and the TNF-α/IL-6 loci depend on different TLR downstream signalling. Upon TLR ligation IRF-7, which is constitutively expressed by resting pDCs, translocates into the nucleus to initiate type I IFN transcription5. On the other hand, IRF-5 nuclear translocation together with NF-κB and MAPK activation, is crucial for the production of inflammatory cytokines6.

Upon crosslinking, BDCA2 signals via a signaling pathway resembling that of the B cell receptor (BCR) inducing tyrosine phosphorylation and Src kinase dependent calcium influx31 and phosphorylation of Syk, SLP65, PLCγ2 and cytoskeletal proteins30,33. Also crosslinking of ILT7 results in ITAM-mediated signaling and phosphorylation of Src family kinases and Syk kinases and induces calcium influx in pDCs29. Interestingly Spi-B has been implicated in the regulation of genes important for BCR signaling37. It is therefore possible that Spi-B enhances signaling through BDCA2 or ILT7 which may provide an explanation how high Spi-B levels prevent IFN-α, IL-6 and TNF-α production. Still, another mechanism of action for Spi-B in pDCs must be proposed that accounts for the effect of Spi-B on the expression of maturation markers, as the induction of CD80 and CD86 expression upon pDC stimulation is not affected by cross-linking BDCA2 or ILT7. Like the secretion of IL-6 and TNF-α, expression of co-stimulatory molecules probably also depends on IRF-5 nuclear translocation and NF-κB and MAPK activation5. Further research is required to reveal if and how Spi-B interferes with TLR downstream signaling.

Upon stimulation pDCs produce type I IFNs and proinflammatory cytokines and differentiate into antigen-presenting mature pDCs (Figure 6). In experimental systems, the effector phenotype of pDCs can be controlled by the type of stimulation. Triggering of CD40/IL-3R on
pDCs leads to the induction of mature pDCs that produce relatively low levels of type I IFN. TLR9 stimulation by CpG selectively induces type I IFN production and differentiation into a mature pDCs, depending on the structure and intracellular location of CpG. Activation of TLR9 by the multimeric CpG-A (CpG 2216) occurs in endosomes and leads exclusively to type I IFN production, whereas the monomeric CpG-B (CpG 2006) localizes to the lysosome and promotes CD80 and CD86 expression of pDCs.

In the current view of pDC differentiation in response to microbial or viral stimulation the two effector stages occur sequentially. Under physiological conditions pDCs have a plasmacytoid morphology with a well-developed ER, i.e., already in steady state pDCs are fully mature effector cells of the innate immune system, corresponding to the professional type I IFN-producing cells. After producing large amounts of type I IFN in response to microbial or viral stimulation pDCs undergo morphological changes to form dendrites and upregulate the surface expression of co-stimulatory and MHC molecules. A recent report introduces the model of alternative pathways in pDC differentiation, i.e., dependent on stimulation, pDCs either differentiate into cells that produce large amounts of type I IFN or differentiate into cells that produce little or no IFN-α but efficiently process and present antigen. After pDCs have been induced to produce IFN-α by CpG-A stimulation, they can be induced to upregulate CD86 expression by subsequent CpG-B stimulation and vice versa. This strongly suggests that both events are induced mutually exclusively rather than consecutively.

Changes of expression levels of factors involved in pDC differentiation could give important clues to justify one of the models. Currently, our data on how Spi-B regulates functional differentiation of pDCs by Spi-B does not preferentially support one of the two models. We observed that Spi-B protein levels were decreased within 24h upon activation of ex vivo isolated pDCs independent of the mode of stimulation (Figure 1). We did not observe different expression of Spi-B after stimulation of pDCs with CpG 2216 or CpG 2006, respectively. This finding is not consistent with our model in which Spi-B plays a crucial role in determining the effector fate of pDCs, as it implies that various stimuli inducing alternative effector fates would differentially alter Spi-B protein levels. We speculate that the observed downregulation of Spi-B protein is required for optimal cytokine production, for example upon stimulation with CpG 2216. However, the hyperactivated pDC phenotype we have observed upon Spi-B overexpression cannot be linked to endogenous Spi-B downregulation upon stimulation. We only assessed Spi-B levels within the total pDC population and not in single cells. As intracellular IFN-α stainings suggest that only a portion of pDCs produce cytokines upon stimulation, it would be interesting to assess Spi-B levels in IFN-α-producers versus non-producers.

Figure 6. Spi-B regulates the functional differentiation of pDCs. Spi-B is required for the development of pDCs from progenitors. Upon microbial or viral stimulation pDCs produce IFN-α and pro-inflammatory cytokines, and differentiate into antigen-presenting mature pDCs by changing morphologically and upregulating migratory, co-stimulatory and MHC molecules. Our data suggest a role for Spi-B in pDC maturation towards a mature phenotype while preventing the ability of pDCs to produce IFN-α, TNF-α and IL-6. HSC: Hematopoietic stem cell, IPC: IFN-producing cell.
The physiological relevance of the regulation of the expression of surface molecules in pDCs required for antigen presentation remains to be determined as it is not well established if pDCs have the capacity to prime naïve T cells in vivo. Several studies with human and mouse pDCs show that in vitro activated pDCs are able to induce expansion of memory CD8+ T cells and Th1 CD4+ T cells specific for endogenous antigens and influenza virus, present pulsed peptides to naïve T cells and induce a potent Th1 polarization, and expand naïve CD8+ T cell populations in vivo in response to endogenous and exogenous antigens, respectively. More recent data in mice addressing the in vivo potential of unmanipulated pDCs to prime naïve T cells show that pDCs can initiate productive naïve CD4+ T cell responses in lymph nodes, but not in the spleen, without concomitant CD8+ T cell priming unlike in cDC-driven responses. To which extend pDCs contribute to first line antigen presentation in a cDC-competent host has not yet been established.
References


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Supplementary figures

Figure S1. Spi-B is exclusively expressed in pDCs, not in cDCs. pDCs and cDCs were isolated by flow cytometry from PNT or PB, or generated from CD34+CD1a progenitors in OP9-cocultures. The cells were analyzed for Spi-B expression by quantitative PCR. Values are normalized to expression in cDCs. Averages ± SD of PCR duplicates are shown. One representative experiment out of three is shown.

Figure S2. Upregulation of CD40 and CD86 expression upon stimulation of pDCs. CD123hiCD45RA+ pDCs were isolated from PNT and analyzed directly or stimulated overnight as indicated. Surface expression levels of CD40 and CD86 were assessed by flow cytometry. Mean fluorescence intensities (MFI) are shown. One representative experiment out of three is depicted.

Figure S3. Stimulation of pDCs generated in vitro. CD34+CD1a progenitors isolated from PNT were cultured on OP9 cells for 7 days. Bulk cultures were stimulated overnight with CpG 2216 and analyzed for expression of CD123 and CD40 by FACS. Gates indicate the CD123hi pDC population. One representative experiment out of seven is shown.

Figure S4. Knock-down of Spi-B by shRNA. CAL-1 cells were transduced with Spi-B shRNA GFP or control construct targeting an irrelevant gene product (Renilla) and sorted for GFP expression. Lysates were analyzed for Spi-B protein and, as loading control, actin.