Transcriptional control of human plasmacytoid dendritic cell and B cell differentiation
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Development of human plasmacytoid dendritic cells depends on the combined action of E2-2 and Spi-B

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Abstract

Plasmacytoid dendritic cells (pDCs) are central players in the innate and adaptive immune response against viral infections. The molecular mechanism that underlies pDC development from progenitor cells is only beginning to be elucidated. Previously we reported that the Ets-factor Spi-B and the inhibitors of DNA binding protein 2 (Id2) or Id3, which antagonize E-protein activity, are crucially involved in promoting or impairing pDC development, respectively. Here we show that the basic-helix-loop-helix (bHLH) protein E2-2 is predominantly expressed in pDCs, but not in their progenitor cells or conventional DC (cDC). Forced expression of E2-2 in progenitor cells stimulated pDC development. Conversely, inhibition of E2-2 expression by RNA interference impaired the generation of pDCs suggesting a key role for E2-2 in development of these cells. Notably, Spi-B was unable to overcome the Id2 enforced block in pDC development and moreover Spi-B transduced pDCs expressed reduced Id2 levels. This might indicate that Spi-B contributes to pDC development by promoting E2-2 activity. Consistent with this notion simultaneous overexpression of E2-2 and Spi-B in progenitor cells further stimulated pDC development. Together our results provide additional insight into the transcriptional network controlling pDC development as evidenced by the joint venture of E2-2 and Spi-B.
Introduction

The ability of dendritic cells (DCs) to capture and present antigenic peptides, has established a function in both the adaptive and innate branches of the immune system. Extensive characterisation of DCs has revealed the existence of many different DC subsets with distinct cell surface phenotype, cytokine expression profile, and anatomical localisation. One member of the DC family is the plasmacytoid DC (pDCs), which is hallmarked by their capacity to produce high levels of type I interferons (IFN) and hence are also known as natural type I IFN producing cells. pDCs are detected in blood and most tissues, including spleen, lymph nodes and thymus. Previously, we described that at least two developmental pathways exist for pDCs, an extrathymic and an intrathymic pathway. The requirements for the development of DC subsets are not fully understood. In mice it has been shown that conventional DCs (cDC) and pDCs can develop from minor Flt3+ subpopulations within the common myeloid and the common lymphoid progenitor pools. Recently, this pool was narrowed down to a DC committed precursor (pro-DC) that can only develop into cells of the DC lineages. It is not clear yet at what point in DC development the commitment to a subpopulation is accomplished. Also human pDCs can be derived from both myeloid and lymphoid progenitor cells. A better understanding of the molecular mechanisms that control DC development may contribute to the elucidation whether one or more developmental pathways of pDCs exist. Studies with gene targeted mice have revealed several transcription factors implicated in pDC development, including STAT3, which is involved Flt3L-dependent dendritic cell differentiation, Ikaros, interferon regulatory factor (IRF)-4 and IRF-8, Gfi1, and XBPI. Deficiency in some of these factors results in specific ablation of just the pDC subset, whereas others additionally affect the development of lymphoid-tissue resident or skin DCs. Our lab has implicated a crucial role for the Ets transcription factor Spi-B in human pDC development. Forced expression of Spi-B in CD34+ progenitor cells favoured pDC development but impaired the development of B, T, and, natural killer (NK) cells. More importantly, reducing expression of Spi-B by means of RNA interference or triggering of the Notch1 pathway strongly inhibited the development of pDCs both in vitro and in vivo. Furthermore we have provided evidence for a role for E-proteins in pDC development. E-proteins are a class of four proteins (TCF12/HEB, TCF4/E2-2, and the E2A splice variants E12 and E47), which are members of the bHLH superfamily of transcription factors. The involvement of E-proteins in pDC development was deduced from experiments with inhibitors of DNA binding (Id) proteins. Like E-proteins the Id-factors also harbour a helix-loop-helix domain for protein-protein interactions but lack the basic DNA binding domain, thereby restraining E-protein activity upon complex formation. Inhibition of E-protein activity by forced expression of Id2 or Id3 in CD34+ progenitor cells inhibited the development of pDCs, but not that of cDCs. Consistent with this, mice lacking Id2 have increased percentages of pDCs. Together this suggests that one or more E-proteins are required for the development of pDCs.

Here we identified E2-2 as a crucial factor in human pDC development. E2-2 is highly expressed in pDCs, but not in their progenitor cells or in cDCs. Overexpression of E2-2 stimulated the differentiation of thymic CD34+CD1a- progenitor cells into pDCs. Conversely, inhibition of E2-2 expression by RNA interference impaired pDC development. This identifies E2-2 as another key player in development of this cell lineage. Spi-B is unable to overcome the block in pDC development imposed by Id2. Furthermore, Spi-B transduced pDCs express reduced levels of Id2. This together with the observation that E2-2 and Spi-B, when overexpressed, simultaneously further enhanced pDC development suggests that the concurrent action of Spi-B and E2-2 controls the development of progenitor cells into the pDC lineage.
Materials and methods

Monoclonal antibodies. Monoclonal antibodies to CD3, CD4, CD34, CD45RA, CD56, CD123 and HLA-DR conjugated to PE, PerCP, PeCy7, APC or APC-Cy7 were purchased from Becton Dickinson (BD, San Jose, CA), CD1a-PE from Beckman Coulter (Fullerton, CA), BDCA2-APC from Miltenyi Biotech (Bergisch Gladbach, Germany), and CD56-APC from Beckman Coulter (BC, Marseille, France).

Constructs, cell lines and retrovirus production. The retroviral constructs, LZRS Spi-B IRES GFP and LZRS Id2 IRES GFP were described previously. Spi-B and Id2 were subcloned into LZRS IRES YFP by using restriction enzymes XhoI-NotI and NotI (Roche, Germany) respectively. PCR was performed to obtain human E2-2 cDNA from thymic pDCs by using 2 primer sets

set 1:
GTGTCTGGGACCTGATCGGG
CAGGAGGCGTACAGGAAGAG

set 2:
CTTGCGTCTGGGATTCATAAC
GCCTGGCTATGCAGGAATGT

TOPO TA Cloning kit (Invitrogen, CA) was used to ligate the PCR products into pCR2.1-TOPO vector. The inserts were then liberated by EcoRI and BstXI and subcloned into the EcoRI site of LZRS ires GFP. Control and Spi-B RNA-i constructs were described previously20. The RNA-i sequence (5'-TCGCAGACGCAAGAGGTTT-3') specifically targeting human E2-2 mRNA was designed using Ambion's siRNA Target Finder (http://www.ambion.com). Using those constructs, GALV-pseudotyped retroviruses were produced using the Phoenix packaging cell line.

Isolation of CD34+ cells, pDCs, and cDCs from postnatal thymus and buffy coat. Buffycasts for isolation of pDCs and cDCs were obtained from Sanquin Bloodbank, Amsterdam, the Netherlands. Thymocytes were obtained from surgical specimens removed from children up to 3 years of age undergoing open heart surgery, with informed consent from patients in accordance with the Declaration of Helsinki and approved by the Medical Ethical Committee of the Academic Medical Center. Thymocytes and peripheral blood lymphocytes were isolated from a Ficoll-Hypaque density gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway). Subsequently, CD34+ cells were enriched by immunomagnetic cell sorting, using a CD34 cell separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD34+CD1a+CD56+BDCA2+ cells, further referred to as CD34+CD1a+, were sorted to purity on a FACSAria (BD). Thymic and peripheral blood BDCA4+ and HLA-DR+ cells were isolated by MACS cell separation. CD123+CD45RA- pDCs and CD3+CD56+CD19+ HLA-DR+CD11c+ cDCs were further separated by cell sorting.

Retroviral transduction and differentiation assays. For transduction experiments CD34+CD1a+ postnatal thymocytes were cultured overnight with 20 ng/mL SCF (R&D Systems), and 10 ng/mL IL-7 (PeproTech) and subsequently incubated for 6 hours with virus supernatant in retronectin coated plates (30 μM; Takara Biomedicals, Otsu, Shiga, Japan). 1×10⁵ CD34+CD1a+ progenitor cells were co-cultured with 5×10⁵ OP9 cells in MEM (Invitrogen) with 20% FCS (Hyclone), 5 ng/mL IL-7 and 5 ng/mL Flt3L (PeproTech). Differentiation assays were analyzed after 1 week of co-culture. Flow cytometric analysis was performed on an LSRII FACS analyzer (BD). To obtain in vitro generated pDCs and cDCs, CD123+BDCA2+ pDCs and CD3+CD56+CD19+ HLA-DR+CD123+CD11c+ cDCs were isolated from co-culture after 10 days by FACS sorting.

Quantitative real-time PCR. Quantitative real-time PCR was performed with an iCycler (Bio-Rad, Hercules, CA), using specific primers. Expression levels relative to actin expression were calculated. Actin, forward ATGGAGTTGGAAGGATAGTTGG
Actin, reverse CAAGAGATGGCCAGGCTGTCAGC
E12, forward ACAGCGAGAAGCCCCAGA
E12, reverse CTGGCTTTGGGATTCAGGTTC
E47, forward GTCGGACAAAGCGCAGAC
E47, reverse ACAGGCTGCTTTGGGATTC
HEB, forward CCGTGGCAGTATCGAATTC
HEB, reverse GCCGATACGGCAAAACCT
E2-2, forward ATGGGAGAGAATCAAACTTA
E2-2, reverse CCTCCATGGCACTACTGTGA
Id2, forward CCGGATTACATCCTGGTCC
Id2, reverse CTGAATAAGCGGGTGTTCATGA

Immunoblotting. E-protein expression in human thymocytes was assessed by immunoblot analysis using mouse monoclonal E2-2 antibody (ab32873, Abcam, CA), rabbit polyclonal HEB antibody (sc-357, Santa Cruz Biotechnology, CA) and mouse monoclonal E2A antibody (sc-416, Santa Cruz Biotechnology, CA), respectively.
Chapter 2

Results

The bHLH factor E2-2 is highly expressed in pDCs.

Mice lacking expression of the inhibitor of DNA binding Id2 have increased percentages of pDCs. In line with this, forced expression of Id2 and Id3 in human progenitor cells specifically inhibits pDC differentiation, while leaving the development of cDCs unaffected. Id factors are known for their ability to directly restrain the transcriptional activity of E-proteins (HEB, E2-2, and the E2A splice-variants E12 and E47) by protein-protein interaction. Consequently, this suggests the involvement of one or more E-proteins in pDC development. We set out to reveal the E-protein(s) involved in human pDC development. To determine the E-proteins expressed in pDCs, CD123hiCD45RA+ cells were sorted from human postnatal thymus tissue (Supplementary Figure 1A). CD34+CD1a− thymic progenitors, known to have pDC potential and T cell committed CD34+CD1a+ progenitors, were sorted concomitantly for comparison (Supplementary Figure 1B). At the mRNA level the E2A splice variants E12 and E47 were detected in all subsets, the CD34+CD1a− and CD34+CD1a+ progenitor cells and pDCs (Figure 1A). However, while E12 and E47 protein was detected in the Jurkat T cell line, these were absent from the sorted thymocyte subsets (Figure 1B). In line with its role in human T cell development (unpublished data R.S. and B.B.), HEB was expressed at higher mRNA and protein levels in the CD34+CD1a− than in the CD34+CD1a+ thymic progenitor population. However, HEB levels were reduced in pDCs (Figure 1A and 1B). In sharp contrast, E2-2 was highly expressed in pDCs both at the transcriptional and protein level, while only low levels were present in CD34+CD1a− or CD34+CD1a+ thymic progenitors (Figure 1A and 1B). E2-2 expression is not limited to pDCs in the thymus, since also pDCs isolated from peripheral blood expressed E2-2 (Figure 1C).

Figure 1. Ex vivo isolated and in vitro generated pDCs, but not cDCs, express high E2-2 levels. (A+B) Freshly isolated CD34+CD1a− and CD34+CD1a+ progenitor cells and CD123+CD45RA+ pDCs from human postnatal thymus were analyzed for the expression of E-proteins by (A) real time RT-PCR and (B) immunoblotting. Lysates from Jurkat cells were analyzed as positive control for E12 and E47 protein detection. Actin levels were determined as loading control. (C) E2-2 expression was determined in ex vivo (from postnatal thymus and peripheral blood) and in vitro generated pDCs and cDCs by real-time RT-PCR. Values are normalized to expression (A) in CD34+CD1a− cells or (C) in cDCs. The error bars represent SDs of triplicate PCR samples. All experiments shown are one representative of three.
In CD11c^{+}HLA-DR^{+} cDCs either isolated from the thymus or peripheral blood E2-2 was not detectable. PDCs and CD11c^{+}HLA-DR^{+} cDCs can also be generated in vitro from progenitor cells after culture on OP9 cells in the presence of Flt3L (21 and H.S. unpublished observations). In line with expression in the ex vivo DC subsets from thymus and peripheral blood we observed that the in vitro generated pDCs, but not cDCs, expressed E2-2 (Figure 1C). Expression levels of the other E-proteins, E12, E47, and HEB, in cDCs were even lower compared to pDCs (data not shown).

In summary, E2-2, but not any of the other E-proteins, is highly expressed in pDCs as compared to their progenitor cells. Notably, E2-2 expression is restricted to pDCs as it is not expressed in cDCs. Furthermore E2-2 expression is independent of the pDC localization (thymus or periphery).

**E2-2 is required for the development of pDCs.**

The high expression of E2-2 in pDCs relative to their progenitor cells prompted us to address whether E2-2 has a role in the development of these cells. To investigate this we generated a retroviral construct to overexpress the E2-2 cDNA together with green fluorescent protein (GFP) as a marker gene. E2-2 overexpression was confirmed by immunoblotting on a total lysate of 293T cells that were transfected with the E2-2 construct (Figure 2A). To establish the role for E2-2 in pDC development E2-2 was forced in CD34^{+}CD1a^{−} thymic progenitor cells by retroviral transduction. Both transduced and non-transduced CD34^{+}CD1a^{−} postnatal thymocytes were co-cultured with the murine stromal cell line OP9 and the cytokines IL-7 and Flt3L, a condition regimen known to support the development of pDCs in vitro. After 7 days the cultures were analyzed by flow cytometry for the presence of transduced BDCA2^{+}CD123^{+} pDCs.

**Figure 2. E2-2 stimulates pDC development in vitro.**

(A) E2-2 protein levels in 293T cells transfected with the LZRS E2-2 IRES GFP or empty control vector. Total cell lysates were analyzed by immunoblotting. Actin staining was used as loading control. (B-D) CD34^{+}CD1a^{−} thymic progenitor cells were retrovirally transduced with the LZRS E2-2 IRES GFP or control LZRS IRES GFP vector. After 7 days of co-culture with OP9 cells and IL-7 plus Flt3L, the cultures were analyzed by flow cytometry using antibodies directed against the pDC markers CD123 and BDCA2. (B) Percentages of E2-2 and control transduced pDCs obtained in a representative experiment are shown after electronic gating on GFP^{+} cells. (C) Normalized percentages of GFP^{+} pDCs obtained after E2-2 or control transductions. The percentage of pDCs in the control cultures is set to 100%. (D) Normalized absolute numbers of GFP^{+} pDCs, calculated based on the input of progenitor cells and expansion rate of GFP^{+} pDCs. Values are normalized to control transduced pDCs, which is set as 1. Averages ± SD of 9 experiments are shown. **p-value<0.01; *p-value <0.05.
The percentages of transduced cells in this representative experiment were 4% and 3%, while the total cell numbers were $1.6 \times 10^6$ and $2.3 \times 10^6$ for control and E2-2 transduced cultures, respectively. Increased E2-2 expression significantly enhanced the development of pDCs from progenitors, as demonstrated in both percentages and absolute cell numbers (Figure 2B-D), indicating that E2-2 promotes pDC differentiation.

**Knockdown of E2-2 expression by RNA interference impairs pDC development.** To more firmly establish the role of E2-2 in pDC development we employed a RNA interference approach to knockdown E2-2 expression. Retroviral pRetrosuper vectors were constructed to express the E2-2 or Renilla control RNA-i sequences together with the marker gene yellow fluorescent protein (YFP) driven by an independent PGK promoter. One of the three probes that we designed to target the E2-2 mRNA (E2-2i #3) for degradation resulted in an almost 60% inhibition of E2-2 protein expression in the 293T cells in comparison to control Renilla RNA-i transfected 293T cells (Figure 3A).

Figure 3. pDC development is impaired by knocking down E2-2 expression. (A) E2-2 protein levels in 293T cells transfected with either E2-2 RNA-i probes or as control Renilla RNA-i probe as determined by immunoblot analysis. Actin was used as a loading control. Ratios (E2-2:Actin) are normalized to control Renilla-i. (B-D) CD34$^+$CD1a$^-$ thymic progenitor cells were retrovirally transduced with pRetrosuper (E2-2 RNA-i/pgk YFP) or pRetrosuper (Renilla RNA-i/pgk YFP) vectors and co-cultured with OP9 cells plus IL-7 and Flt3L. After 7 days the cultures were analyzed by flow cytometry using antibodies directed against the pDC markers CD123 and BDCA2. (B) A representative experiment showing percentages of E2-2 RNA-i (E2-2i) and control Renilla RNA-i (Renilla-i) transduced pDCs after electronic gating on YFP$^+$ cells. (C) Normalized percentages of YFP$^+$ pDCs obtained after E2-2-i or control Renilla-i transductions. The percentage of pDCs in the control cultures is set to 100%. (D) Normalized absolute numbers of YFP$^+$ pDCs, calculated based on the input of progenitor cells and expansion rate of YFP$^+$ pDCs. Values are normalized to control transduced pDCs. Averages ± SD of 6 experiments are shown. **p-value < 0.01; *p-value < 0.05.
Then, to determine whether pDC development is dependent on E2-2 expression, CD34^+CD1a^- thymic progenitor cells were transduced with either the E2-2 RNA-i/pgk YFP or Renilla RNA-i/pgk YFP knockdown constructs and cultured under pDC promoting conditions on OP9 cells with IL-7 and Flt3L. After 7 days of co-culture, the percentages of transduced cells in this representative experiment were 10% and 9%, while the total cell numbers were 1.6x10^6 and 1.2x10^6 for Renilla-i and E2-2-i transduced cultures, respectively. We observed a reduction in the percentage as well as in the absolute cell number of pDCs when E2-2 levels were decreased by means of RNA interference (Figure 3B-D). This, together with the enhanced development when E2-2 expression is increased, argues for an important role of E2-2 in human pDC development.

**Spi-B cannot stimulate pDC development in absence of E2-2 activity.**

Forced expression of Id2 and Id3 in human progenitor cells inhibits pDC differentiation. Blocking pDC development by forced expression of Id2 could be relieved to control transduced levels by concomitant expression of E2-2 (Supplemental figure 2), confirming the reciprocal functions of these factors in our system. Previously, we have reported that the Ets transcription factor Spi-B is crucial for the development of pDCs from progenitor cells. We aimed to determine the relative contribution of E2-2 and Spi-B in progenitor cells developing into the pDC lineage. To this end we assessed the effect of overexpressing Spi-B while concomitantly knocking down E2-2 expression in thymic progenitor cells and observed that the enhanced pDC development induced by Spi-B was diminished when also E2-2i#3 was overexpressed (data not shown).

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**Figure 4. Spi-B does not overcome the block in pDC development induced by Id2.** CD34^+CD1a^- thymic progenitor cells were retrovirally transduced with Id2/GFP, Spi-B/YFP, or control transduced with GFP and/or YFP only vectors. After 7 days of co-culture with OP9 cells in the presence of IL-7 and Flt3L, the cultures were analyzed by flow cytometry using antibodies directed against the pDC markers BDCA2 and CD123. (A) Shown are the percentages of pDCs after gating on double transduced GFP^+YFP^+ cells from a representative experiment. The percentages of double transduced cells in this experiment were as follows: 0.13% control-GFP/control-YFP, 0.24% control-GFP/Spi-B YFP, 0.65% Id2 GFP/control YFP, 0.92% Id2 GFP/Spi-B YFP. (B) Normalized percentages of GFP^+YFP^+ pDCs in the OP9 cultures. The percentage of pDCs in the control cultures is set to 100%. (C) Normalized absolute numbers of GFP^+YFP^+ pDCs, calculated based on the number of GFP^+YFP^+ transduced input progenitor cells and the number of GFP^+YFP^+ BDCA2^+CD123^+ pDCs after 7 days of co-culture. Values are normalized to control transduced pDCs. Averages ± SD of 3 experiments are shown. **p-value<0.01; *p-value <0.05. ns: not significant.
This reduction was comparable to the condition where we only knocked down E2-2 expression. Since it was difficult to correctly interpret these data, due to the fact that knocking down E2-2 expression by RNA interference is incomplete, we decided to inhibit E-protein activity by expression of Id2 in conjunction with Spi-B. Progenitor cells were double transduced with Id2/GFP and Spi-B/YFP constructs and co-cultured with OP9 cells. After one week of culture the GFP+YFP+ double transduced cells were analyzed for the presence of BDCA2⁺CD123⁺ pDCs (Figure 4). As expected, when only Spi-B was overexpressed in the progenitor cells we observed an increase in both the percentage and absolute cell number of pDCs (Figure 4A-C). Conversely, Id2 overexpression significantly inhibited pDC development as compared to control transduced cells. Notably, when both Spi-B and Id2 were overexpressed simultaneously neither the percentage nor the absolute pDC numbers increased compared to the Id2 only transduced cultures (Figure 4A-C). Taken together this indicates that Spi-B is unable to direct progenitor cells into the pDC lineage in the presence of the E-protein inhibitor Id2. The functional activity of bHLH factors, most likely E2-2, is an absolute requirement for development of pDCs. This further emphasizes the importance of E2-2 in pDC development.

**E2-2 cannot stimulate pDC development in absence of Spi-B activity.**

To further establish the notion that both E2-2 and Spi-B are required for pDC development, we assessed the effect of E2-2 overexpression in progenitor cells in which Spi-B levels were reduced. Previously we have reported that pDC development is inhibited when expression of Spi-B is abrogated by RNA interference in progenitor cells. We employed the knockdown construct to impair Spi-B expression simultaneous with E2-2 overexpression in thymic progenitor cells and analyzed the effect on pDC development after co-culture with OP9 cells. As expected, after one week we observed that E2-2 overexpression increased pDC development, while knocking down Spi-B expression reduced both the percentage and absolute pDC numbers (Figure 5A-C). Notably, in the condition that E2-2 was overexpressed but Spi-B expression was reduced we observed that pDC development was impaired compared to E2-2 overexpression only. The percentages and absolute cell numbers, respectively, of pDCs in this experiment were as follows: Control GFP + Renilla-i YFP (24%, 2738 pDCs), Spi-B GFP + Renilla-i YFP (56%, 3796 pDCs), Control GFP + E2-2-i YFP (15%, 1006 pDCs) and Spi-B GFP + E2-2-i YFP (34%, 2924 pDCs). Together these data suggest that E2-2 is unable to stimulate pDC development in the absence of Spi-B and confirms that both proteins are required for proper pDC differentiation.

**Spi-B regulates the expression of Id2.**

The finding that Spi-B overexpression did not overcome the Id2 enforced block in pDC development (Figure 4) let us to speculate that the regulation of Id proteins may be a mechanism of Spi-B in promoting pDC lineage development. This notion would be in line with the data presented in Figure 5 that show requirement for adequate Spi-B protein levels in the cells to allow for E2-2-mediated pDC development. To investigate whether Spi-B may be involved in regulating the expression of Id2, pDCs were generated in vitro from Spi-B transduced CD34⁺CD1a⁻ precursors and Id2 levels were assessed (Figure 6). We observed that Spi-B transduced pDCs expressed 2-fold lower levels of Id2 as compared to control transduced pDCs. An effect on E2-2 expression by Spi-B was not observed (data not shown). These data may suggest that Spi-B is involved in the regulation of Id transcription and thereby may indirectly enhance E2-2 activity and promote pDC development.
E2-2 and Spi-B cooperate in pDC development

Figure 5. Reducing Spi-B levels impairs E2-2 induced pDC development. CD34⁺CD1a⁻ thymic progenitor cells were retrovirally transduced with Spi-B RNA-i/GFP (Spi-B-i), E2-2 IRES YFP and/or the appropriate control vectors. After 7 days co-culture with OP9 cells in the presence of IL-7 and Flt3L, cultures were analyzed by flow cytometry by using antibodies directed against the pDC marker BDCA2 and CD123. (A) Shown are the percentages of pDCs after electronic gating on double transduced GFP⁺YFP⁺ cells from a representative experiment. The percentages of double transduced cells in this representative experiment were as follows: 1% control-GFP/control-YFP, 0.6% Spi-Bi GFP/control-YFP, 0.2% control GFP/E2-2 YFP, 0.2% Spi-Bi GFP/E2-2 YFP. (B) Normalized percentages of GFP⁺YFP⁺ pDCs in the OP9 cultures. The percentage of pDCs in the control cultures is set to 100%. (C) Normalized absolute numbers of GFP⁺YFP⁺ pDCs, calculated based on the number of GFP⁺YFP⁺ transduced input progenitor cells and the number of GFP⁺YFP⁺ BDCA2⁺CD123⁺ pDCs after 7 days of co-culture. Values are normalized to control transduced pDCs, which is set as 1. Averages ± SD of 4 experiments are shown. **p-value<0.01; *p-value <0.05.

Figure 6. Expression of Id2 is reduced when Spi-B is overexpressed. Expression levels of Id2 were assessed by real time RT-PCR in sorted GFP⁺ pDCs derived in vitro from CD34⁺CD1a⁻ progenitors transduced with Spi-B or control vectors. Values are normalized to expression in control transduced cells. Averages ± SD of PCR duplicates are shown. One representative experiment out of two is shown.
Co-expression of E2-2 and Spi-B stimulates pDC development.

If Spi-B controls pDC development by downregulating the expression of Id factors and thereby releases the antagonistic effect on E2-2 activity we might expect that overexpression of both Spi-B and E2-2 will further enhance the pDC differentiation compared to cells overexpressing either one of the two transcription factors individually. To test this hypothesis CD34^+CD1a^- progenitor cells were double transduced with constructs expressing Spi-B and E2-2 and co-cultured with OP9 cells. As shown in Figure 7, after one week of culture a significantly higher percentage and absolute number of E2-2/Spi-B double transduced cells had developed into pDCs compared to control transduced or E2-2 only transduced cultures. From this we conclude that E2-2 and Spi-B act in a cooperative manner to stimulate the development of human pDCs.

Figure 7. Co-expression of E2-2 and Spi-B stimulates pDC development. CD34^+CD1a^- thymic progenitor cells were retrovirally transduced with E2-2/YFP and/or Spi-B/GFP and/or the appropriate control vectors. After 7 days coculturing with OP9 in the presence of IL-7 and Flt3L, the cells were analyzed by flow cytometry by using antibodies directed against the pDC marker CD123 and BDCA2. (A) Shown are the percentages of pDCs after electronic gating on double transduced GFP^+YFP^+ cells from a representative experiment. The percentages of double transduced cells were as follows: 1.8% control-GFP/control-YFP, 0.6% Spi-B GFP/control YFP, 0.4% control GFP/E2-2 YFP, 0.2% Spi-B GFP/E2-2 YFP. (B) Normalized percentages of GFP^+YFP^+ pDCs in the OP9 cultures. The percentage of pDCs in the control cultures is set to 100%. (C) Normalized absolute numbers of GFP^+YFP^+ pDCs, calculated based on the number of GFP^+YFP^+ transduced input progenitor cells and the number of GFP^+YFP^+ BDCA2^+CD123^+ pDCs after 7 days of co-culture. Values are normalized to control transduced pDCs. Averages ± SD of 4 experiments are shown. **p-value<0.01; *p-value <0.05.
Discussion

In this report we identify the E-protein E2-2 as a crucial regulator of human pDC development. By real time RT-PCR and immunoblotting high levels of E2-2 were detected in ex vivo isolated pDCs when compared to progenitor cells or cDCs from human blood or thymus. Overexpression of E2-2 in CD34+CD1a+ progenitor cells strongly promoted pDC development in Flt3L supplemented cell cultures whereas its knockdown effectively reduced the ability of human progenitors to develop into pDCs. An important result emanating from our studies was the observation that Spi-B, an Ets-factor that we previously identified as the key factor required in pDC development, was incapable to overcome the Id2 enforced block in pDC development. This together with the finding that Id2 expression levels were reduced in Spi-B transduced pDCs, may suggest that Spi-B acts to promote E2-2 activity. In line with this, we observed that E2-2 and Spi-B when co-expressed further enhanced the development of pDCs.

E-proteins are essential factors in lymphocyte development and function. In particular, E-proteins are crucial for development of lymphoid progenitors to the B and T cell lineages. In the development of T cells, both E2A and HEB have been implicated. E12 and E47 are essential for B cell development by controlling either directly or indirectly the expression of Pax-5, a factor essential for B cell lineage fate decision and securing of the B cell identity until the mature stages of development. In contrast, HEB is dispensable for B cell development. E2-2−/− mice die around birth, precluding the analysis of loss-of-function effects at later stages of postnatal life. In conditional E2-2-deficient mice E2-2 deficiency leads to a partial block in both B and T lymphocyte development. The development of DC subsets was not assessed in these mice. The findings described in this manuscript reveal a crucial role for E2-2 in human pDC development. Together with the observations that E-proteins are important regulators of lymphoid development suggest a close lineage relationship of pDCs with the T and B cell lineages. Overexpression of the dominant negative transcription factor Id2 or Id3 blocked development of pDCs, but not of cDCs. In line with this, pDC development is enhanced in Id2 deficient mice. Recently a GeneChip analysis on mouse and human leukocytes showed that E2-2 is expressed at high levels in pDCs, while Id2 was high in cDCs. Here we not only confirmed prominent mRNA and protein expression of E2-2 in pDCs, but in addition provide evidence to suggest that Id factors when overexpressed most likely antagonize E2-2 activity in progenitor cells, thereby blocking development into the pDC lineage. Of notice, we and others did not detect high expression of the other E-proteins HEB, E12, or E47 in pDCs. To our surprise we observed, however, that overexpression of the other E-proteins in human progenitor cells promoted in vitro pDC development to some extent (data not shown). While we cannot exclude a role for the other E-proteins in pDC development, our findings may also be attributed to the fact that high levels of HEB, E12, or, E47 bind to endogenously expressed Id proteins. This then may relieve the negative regulation on E2-2, thereby indirectly promoting pDC development. A similar model was proposed for B cell-lineage commitment and expansion, where E2A is the central player and E2-2 and HEB could modulate the pool size of E2A homodimers through a competitive dimerization with Id factors.

Currently, the target genes that are regulated by E2-2 and account for the effect on pDC development are elusive. Transcription factors often exert their function by forming protein complexes for enhanced DNA binding. E-proteins bind to E-box DNA elements either as homodimers or heterodimers with other bHLH proteins. The consensus E-box sequence (CANNTG) has been identified in a number of regulatory elements of lymphoid lineage specific genes, including the T cell receptor α and γ enhancers, the CD4 silencer and enhancer, and the promoters of mb-1, λ5, and pTα, which are involved in either B or T cell development. Furthermore, it is possible that complexes consisting of Ets factors and IRF bind to ETS-IRF composite DNA elements (EICE). In addition, it has been reported that a ternary complex of PU.1, IRF-4, and E47, by binding to an E-box and EICE element, transactivated expression of the CIITA gene, which was required for expression of MHC class II on B cells. Here we describe that E2-2 and Spi-B cooperate in pDC development. Of notice,
both IRF-4 and IRF-8 are highly expressed in human pDCs (42,51,52, and unpublished results H.S., B.B.) and crucially involved in murine pDC development14,16. It is therefore tempting to speculate that Spi-B, in addition to contributing to the downregulation of Id2 expression, may in complex with E2-2 and IRF-4 and/or IRF-8 bind to a juxtaposed E-box and EICE element to promote pDC development. In the in vitro assay that we employed here we did not observe further stimulation of pDC development when IRF-8, E2-2, and Spi-B were co-expressed compared to E2-2 and Spi-B overexpression. Also did we not observe a reduction in pDC development when knocking down IRF-8 expression by RNA interference (unpublished observation H.S., B.B.). This, however, does not exclude a role for IRF-8 in human pDC development as the protein may already be present at high level, whereas Spi-B and E2-2 may be the limiting factors in commitment to the pDC lineage. Alternatively, IRF-4 which is highly expressed in pDCs42 may exhibit a redundant role. This notion is supported by the fact that IRF4/IRF8 double deficient mice have even less pDCs compared to the single knockout mice53.

In conclusion, our study provides important insight in the complex network of transcription factors that controls progenitor cell differentiation and furthers our understanding on the regulation of human pDC development.

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References


**Supplementary figures**

**Figure S1. Cell sorting strategy and purity of thymic subsets.** (A) Thymocytes were enriched for pDCs by positive selection of BDCA4 expressing cells by MACS cell separation followed by cell sorting of CD123⁺CD45RA⁺ pDCs on a FACS Aria. (B) Thymocytes were enriched for progenitor cells using the CD34⁺ MACS kit followed by cell sorting of CD34⁺CD1a⁻ and CD34⁺CD1a⁺ cells on a FACS Aria. Purity of the sorted cells in all experiments was >97%.

**Figure S2. Id2 induced block in pDC development can be overcome by E2-2.** CD34⁺CD1a⁻ thymic progenitor cells were retrovirally transduced with LZRS E2-2 IRES YFP, LZRS Id2 IRES GFP, control LZRS IRES GFP, and/or LZRS IRES YFP vectors. After 7 days of co-culture with OP9 cells in the presence of IL-7 and Flt3L, the cultures were analyzed by flow cytometry using antibodies directed against the pDC markers CD123 and BDCA2. (A) Shown are the percentages of pDCs after electronic gating on double transduced GFP⁺YFP⁺ cells from a representative experiment. The percentages of double transduced cells were as follows: 1.8% control-GFP/control-YFP, 0.8% Id2 GFP/control YFP, 0.4% control GFP/E2-2 YFP, 0.4% Id2 GFP/E2-2 YFP. (B) Normalized percentages of GFP⁺YFP⁺ pDCs in the OP9 cultures. The percentage of pDCs in the control cultures is set to 100%. (C) Normalized absolute numbers of GFP⁺YFP⁺ pDCs, calculated based on the number of GFP⁺YFP⁺ transduced input progenitor cells and the number of GFP⁺YFP⁺ BDCA2⁺CD123⁺ pDCs after 7 days of co-culture. Values are normalized to control GFP⁺YFP⁺ transduced pDCs. Averages ± SD of 3 experiments are shown. **p-value<0.01; *p-value <0.05.