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Brief Definitive Report

The ETS Transcription Factor Spi-B Is Required for Human Plasmacytoid Dendritic Cell Development

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Abstract

A number of transcription factors that act as molecular switches for hematopoietic lineage decisions have been identified. We recently described the ETS transcription factor Spi-B to be exclusively expressed in plasmacytoid dendritic cells (pDCs), but not in myeloid DCs. To assess whether Spi-B is required for pDC development we used an RNA interference knock down approach to specifically silence Spi-B protein synthesis in $CD34^+$ precursor cells. We observed that a knock down of Spi-B mRNA strongly inhibited the ability of $CD34^+$ precursor cells to develop into pDCs in both in vitro assays as well as in vivo upon injection into recombination activating gene $2^{-/-}$ γ $common^{-/-}$ mice. The observed effects were restricted to the pDC lineage as the differentiation of pro–B cells and $CD14^+$ myeloid cells was not inhibited but slightly elevated by Spi-B knock down. Knock down of the related ETS factor PU.1 also inhibited in vitro development of $CD34^+$ cells into pDCs. However, in contrast to Spi-B, PU.1 knock down inhibited B cell and myeloid cell development as well. These results identify Spi-B as a key regulator of human pDC development.

Key words: human plasmacytoid dendritic cells • hematopoiesis • RNA interference • Spi-B

Introduction

DCs are very efficient in inducing adaptive immune responses, but these cells can also be involved in tolerance induction and the regulation of innate immunity. There are different subsets of DCs with distinct cell surface phenotypes, functions, and anatomical localization (1). One member of the DC lineage is the plasmacytoid DC (pDC) precursor (2), also referred to as natural interferon-producing cells (3). pDCs express Toll-like receptors 7 and 9 and have the capacity to produce high levels of type I interferons that block viral replication, indicating that these cells play an important role in innate immunity (4).

The developmental relationship of pDCs with other DC types is unclear. It has been proposed that pDCs originate from lymphoid precursors (5, 6). However, recent findings indicate that pDCs cannot be simply connected to either lymphoid or myeloid lineages (7, 8). Previously we have shown that Flt3L can drive development of human pDCs and myeloid precursors from $CD34^+$ $CD45RA^-$ precursor cells in vitro (9). More recently it has been found in mouse models that the DC precursor activity, including that of pDCs and myeloid DCs, is contained within $Flt3^+$ lymphoid as well as myeloid precursors (7, 8), indicating that a DC developmental program can be induced in both lymphoid and myeloid precursors.

A detailed study of the transcriptional programs that drive DC development may contribute to an understanding of the developmental hierarchy of various DC populations. Various transcription factors have been described to control development of pDCs and other DC populations. Transcription factors that have been implicated are Rel-B (10), PU.1 (11), STAT3 (12), and interferon consensus sequence binding protein (ICSBP)/interferon regulatory factor (IRF)-8 (13). IRF-8 is the only factor identified so far that appears to selectively control pDC development, as mice deficient for this factor lack pDCs but have normal numbers of $CD11b^+$ DCs (13). Recently we have found the ETS family member Spi-B in a genetic search aimed to identify genes specifically expressed in pDCs, but not in $CD14^+$ monocytes and monocyte-derived DCs (14). Spi-B is closely related to PU.1, sharing 67% DNA and 43% overall amino acid sequence identity, but these factors differ in

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tissue expression profile and function. Spi-B is expressed in pDCs, $CD34^+$ precursor cells (14), and in mature B cells, but in contrast to PU.1, not in myeloid cells as monocytes and neutrophils (15). The two ETS factors are also functionally different because in contrast to Spi-B (16), PU.1 is required for development of myeloid cells and B cells (17, 18). We documented that ectopic expression of human Spi-B in $CD34^+$ progenitor cells moderately stimulated development of pDCs in vitro while inhibiting development of T, B, and NK cells (14), strongly suggesting a role of Spi-B in pDC differentiation. However, because the effects of Spi-B inactivation on human pDC development were not analyzed and no information is available about the status of pDCs in Spi-B–deficient mice, the question whether Spi-B is required for pDC development remained unresolved.

In this paper we have applied RNA interference to stably down-regulate Spi-B in $CD34^+$ progenitor cells. Overexpression of short interfering RNA (siRNA) was achieved by retrovirus-mediated transduction using a vector based on the RETROSUPER vector, which was described by Brummelkamp et al. (19). Expression of Spi-B siRNA in $CD34^+$ progenitors resulted in a strong inhibition of pDC development. In contrast, development of B cells and $CD14^+$ myeloid cells was stimulated by Spi-B siRNA.

Materials and Methods

Reagents and Monoclonal Antibodies. Monoclonal antibodies to CD10, CD11c, CD14, CD19, and CD123 conjugated to PE, PerCP, PeCy7, APC, or APCCy7 were purchased from Becton Dickinson. Anti–BDCA2-PE was obtained from Miltenyi Biotec and anti–NGFR-PE was obtained from Chromaprobe. The cytokines IL-7, stem cell factor, and trombopoeitin were obtained from R&D Systems. Flt3L was provided by G. Wagemaker (Erasmus University, Rotterdam, Netherlands). HeLa and Phoenix cells were cultured in Iscove's medium (GIBCO BRL) with 8% FCS. OP9 cells were provided by T. Nakano (Osaka University, Osaka, Japan) and maintained in MEMα (GIBCO BRL) with 20% FCS (20).

Constructs and Retroviral Production. The retroviral construct, LZRS IRES GFP, used to overexpress Spi-B and PU.1 was described previously (14). For knock down experiments, the pSUPER construct described previously by Brummelkamp et al. (19) was adapted. To allow for identification of transduced cells by flow cytometry, a GFP-expressing cassette was added such that the pol3 promoter for transcription of the RNAi probe and the pgk promoter driving GFP expression were positioned in opposite directions. The RNAi sequences specifically targeting either the Spi-B (5′-GATCGCTGTGTGTCTGTAA) or PU.1 (5′-GTCCGTATGTAAATCAGAT) mRNA were designed using Ambion's siRNA Target Finder (http://www.ambion.com). Sequences were inserted into the BglII–HindIII sites of pSUPER GFP. The pol3 RNAi sequence pgk GFP cassette was then subcloned into a self-inactivating derivative of the LZRS retroviral construct (21). The SIN vector was chosen to prevent promoter interference by retroviral promoters in the LTR. For in vitro studies, an RNAi construct targeting the sea pansy (*Renilla reniformis*) luciferase sequence was used as a control (22). For in vivo experiments, control LZRS IRES with a downstream signaling-incompetent mutant of the nerve growth factor receptor (ΔNGFR) and Spi-B RNAi/GFP-transduced progenitor cells were coinjected in SCID RAG-$2^{-/-}$ $\gamma_c^{-/-}$ mice. Using these constructs, GALV-pseudotyped retroviruses were produced using the Phoenix packaging cell line.

RT-PCR. To establish degradation of mRNA by the Spi-B and PU.1 RNAi constructs, an RT-PCR was performed on transfected HeLa cells. 1 μg of the LZRS Spi-B or PU.1 constructs was cotransfected with 10 μg of either the pSIN SUPER Spi-Bi, PU.1i, or Renilla-i knock down constructs described above. 5 d after transfection, RT-PCR was performed on total cDNA. The PCR primers were as follows: Spi-B: 5′-GGAGTGCTGCCCTGCCATAA and 3′-CCCCCACCCCAGATGAGATT; PU.1: 5′-TGG AAG GGT TTC CCC TCG TC and 3′-TGC TGT CCT TCA TGT CGC CG; and HPRT: 5′-TATGGACAGGACTGAACGTCTTGC and 3′-GACACAAACATGATTCAAATCCCTGA.

Isolation of $CD34^+$ Cells from Fetal Liver. Human fetal tissues were obtained from elective abortions. The use of fetal tissue was approved by the Medical Ethical Committee of the Academic Medical Center and was contingent on informed consent. Gestational age was determined by ultrasonic measurement of the diameter of the skull and ranged from 14 to 20 wk. Fetal liver $CD34^+$ cells were isolated as described previously (5).

Retroviral Transduction and Differentiation Assays. Retroviral transductions of $CD34^+$ fetal liver cells were performed as described previously (5). The development of pDCs, pro–B cells, and $CD14^+$ myeloid cells was assessed by coculturing 50,000 $CD34^+$ progenitor cells with 30,000 OP9 cells. Cultures were performed in Yssels medium (23) with 8% FCS supplemented with 5 ng/ml IL-7 and 5 ng/ml Flt3L. Stromal cells and cytokines were refreshed at 7 d of culture.

To study lymphoid development in vivo, sublethally irradiated (350 cGy) neonatal (<1 wk old) RAG-$2^{-/-}$ $\gamma_c^{-/-}$ mice (24) that completely lack T, B, and NK lymphocytes were injected intrahepatically with 10^6 cells containing a 3:2 mixture of Spi-Bi/GFP and control ΔNGFR-transduced $CD34^+$ $CD38^-$ progenitor cells (the transduction efficiencies for both constructs were 15–25%). Humanized RAG-$2^{-/-}$ $\gamma_c^{-/-}$ mice were analyzed at 5–8 wk after injection for the development of human pDCs, B cells, and myeloid cells. Flow cytometric analyses were performed on an LSRII FACS analyzer (Becton Dickinson). All animal experiments were approved by the Animal Experiment Review Board of the Academic Medical Center.

Results and Discussion

Spi-B and PU.1 RNAi Reduce the Amount of Spi-B and PU.1 mRNA, Respectively, and Impair pDC Development In Vitro. We have documented that overexpression of Spi-B in $CD34^+$ precursor cells stimulates differentiation into pDCs (14). To determine whether Spi-B is required for pDC development we used an approach to knock down Spi-B in $CD34^+$ cells. We designed a retroviral construct directing the synthesis of siRNAs to specifically target and degrade the Spi-B mRNA. In parallel we made an RNAi construct for the related transcription factor PU.1. The RNAi constructs were tested for their ability to reduce the amounts of Spi-B and PU.1 mRNA, respectively (Fig. 1 A). To control for introduction of siRNA-expressing constructs we prepared an RNAi construct targeting the nonexpressed Renilla luciferase RNA (22). HeLa cells transfected with

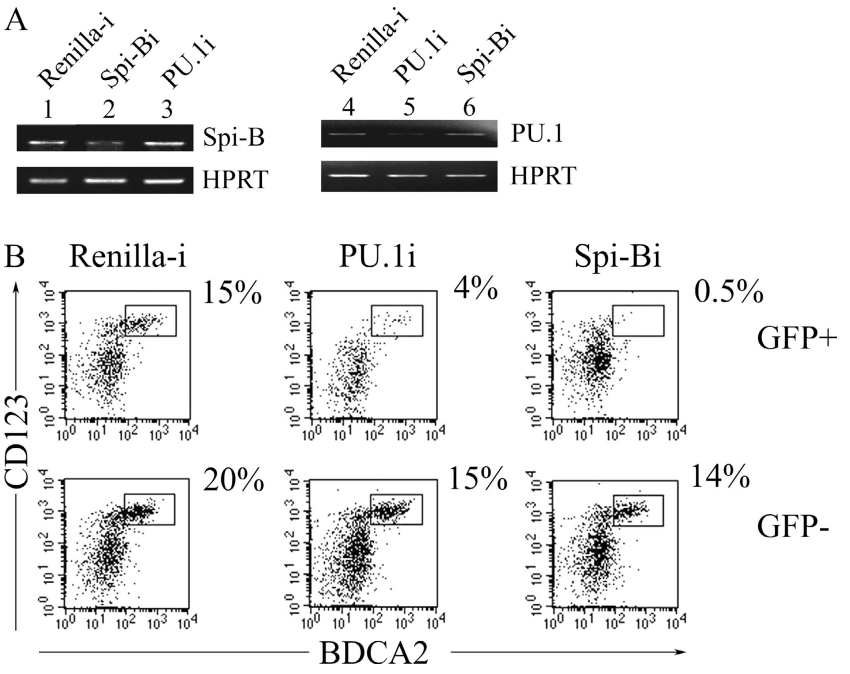

Figure 1. Knock down of Spi-B and PU.1 impair pDC development in vitro. (A) RNA degradation by Spi-B and PU.1 RNAi constructs in transfected HeLa cells. RT-PCR on Spi-B: lane 1, Spi-B plus Renilla-i; lane 2, Spi-B plus Spi-Bi; lane 3, Spi-B plus PU.1i. RT-PCR on PU.1: lane 4, PU.1 plus Renilla-i; lane 5, PU.1 plus PU.1i; lane 6, PU.1 plus Spi-Bi. RT-PCR on the house keeping enzyme HPRT was performed as a loading control. (B) $CD34^+$ $CD38^-$ human fetal liver cells were retrovirally transduced with PU.1i/GFP, Spi-Bi/GFP, or Renilla-i/GFP control virus. Transduced and nontransduced cells were cultured on OP9 cells with IL-7 and Flt3L and analyzed for the development of pDCs by flow cytometry at 7 d of culture. Dot plots represent the transduced (GFP^+) and nontransduced (GFP^-) hematopoietic cell ($CD45^+$) populations. Numbers represent percentages of pDCs as $CD123^{hi}$ $BDCA2^+$ gated events.

the Spi-B overexpression and Spi-B RNAi constructs (Fig. 1 A, lane 2) showed decreased levels of Spi-B mRNA as compared with cells cotransfected with the Renilla-i (Fig. 1 A, lane 1) or PU.1i (Fig. 1 A, lane 3) vectors. This clearly indicates that the Spi-Bi construct is capable of blocking Spi-B protein synthesis. A similar reduction of PU.1 mRNA was observed for cells containing both the PU.1 and PU.1i constructs (Fig. 1 A, lane 5), showing that both constructs are functional.

Having determined the specific down-regulation of Spi-B and PU.1 mRNAs by the RNAi constructs, we tested the effects of Spi-B and PU.1 siRNA on pDC development using an assay described previously (5, 14). $CD34^+$ $CD38^-$ human hematopoietic progenitor cells isolated from fetal liver were transduced with the Renilla, PU.1, or Spi-B siRNA expression constructs. To allow differentiation into pDCs, the mixture of transduced and nontransduced progenitor cells was cocultured with the murine bone marrow stromal cell line OP9 in the presence of IL-7 and Flt3L for 7 d. Within this time frame the cell numbers in these cultures remained the same and thus relative percentages can be considered representative for absolute cell numbers. Fig. 1 B and Table I clearly show that both the PU.1i/GFP^+ and Spi-Bi/GFP^+ progenitor cells are severely impaired in their differentiation toward $CD123^{hi}$ $BDCA2^+$ pDCs as compared with nontransduced cells or cells transduced with the Renilla-i/GFP control construct.

These experiments show that introduction of RNAi-inducing DNA fragments in $CD34^+$ cells using a retroviral vector with the GFP marker allows for expression of siRNAs, which efficiently knock down the genes of interest. We demonstrate that the expression of Spi-B siRNA or

Table I. *Spi-B RNAi Specifically Impairs pDCs and Stimulates Myeloid and B Cell Differentiation In Vitro*

	% $BDCA2^+$ $CD123^{hi}$			% $CD14^+$ $CD11c^+$			% $CD10^+$ $CD19^+$		
Experiment no.	Renilla-i	PU.1i	Spi-Bi	Renilla-i	PU.1i	Spi-Bi	Renilla-i	PU.1i	Spi-Bi
1	6.3	1.5	0.9	13.5	4.6	22.5	2.0	0.1	10.1
2	12.7	1.7	7.1	29.9	22.3	41.5	1.2	0.4	7.5
3	19.1	4.7	1.8	35.1	ND	44.0	18.8	16.3	28.1
4	10.4	5.9	2.9	18.5	ND	23.4	1.1	0.6	13.1
5	10.0	ND	1.7				19.0	ND	31.4
6	3.8	ND	0.8				1.8	ND	3.1
Sample vs. Renilla-i^+ p-value		0.04	0.01		0.05	0.01		0.05	0.00

Statistical analysis on the percentages of pDCs, B cells, and myeloid cells differentiated from $CD34^+$ $CD38^-$ progenitors in the OP9 assay was performed using a paired two-tailed Student's *t* test.

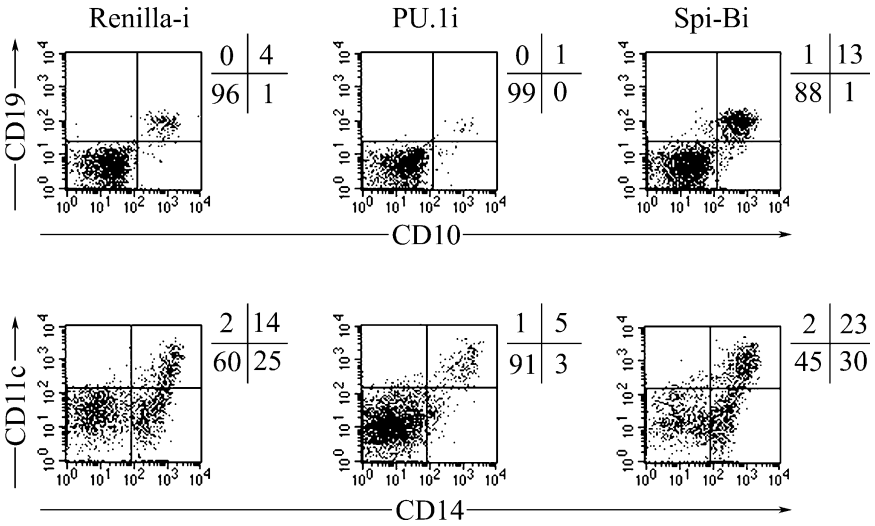

Figure 2. Knock down of Spi-B, but not PU.1, stimulates development of myeloid cells and pro–B cells in vitro. $CD34^+$ $CD38^-$ human fetal liver cells were retrovirally transduced with PU.1i/GFP, Spi-Bi/GFP, or Renilla-i/GFP control virus. Transduced and nontransduced cells were cultured on OP9 cells with IL-7 and Flt3L and analyzed for the presence of myeloid cells and pro–B cells by flow cytometry at 11 d of culture. Dot plots represent the transduced (GFP^+) hematopoietic cell ($CD45^+$) populations. Numbers represent percentages of B cells ($CD10^+$ $CD19^+$) and myeloid cells ($CD11c^+$ $CD14^+$).

PU.1 siRNA in $CD34^+$ cells severely compromised their development into pDCs in vitro. These effects were specific and not due to possible induction of interferon responses because expression of an irrelevant siRNA (Renilla) did not affect pDC development.

Reduced Spi-B Levels Stimulate Myeloid and B Cell Differentiation In Vitro. In addition to a stimulating effect on pDC development, forced expression of Spi-B in human hematopoietic progenitor cells has a strong inhibitory effect on their development into B cells (14). Therefore, we expected that knocking down Spi-B would either not affect or stimulate B cell development. Furthermore, PU.1 is absolutely required for development of murine B cells and also of myeloid cells (18). Therefore, it was of interest to compare the effects of PU.1 and Spi-B siRNAs on development of human B and myeloid cells. In the coculture assay used for pDC development, $CD34^+$ $CD38^-$ fetal liver precursor cells also develop into myeloid and pro–B cells. After 11 d, the coculture assay was analyzed for the presence of myeloid (CD14 and CD11c) or pro–B (CD19 and CD10) cells. Consistent with a developmental block by overexpression of Spi-B, Spi-B knock down led to increased percentages of myeloid and pro–B cells as compared with the nontransduced or Renilla-i/GFP control cultures (Fig. 2 and Table I). The stimulation of myeloid and pro–B cell development was observed only after reduction of Spi-B protein. Reduced levels of PU.1 not only blocked pDCs, but also inhibited myeloid and B cell development, consistent with findings in mouse models that hematopoietic progenitors of $PU.1^{-/-}$ fetal liver cells failed to generate B or myeloid cells (18). Our findings indicate that both Spi-B and PU.1 play a role in human pDC development. Because PU.1 also inhibited development of B cells and myeloid cells, it is likely that the PU.1 knock down inhibits pDC development before the split of B cells, myeloid cells, and pDCs.

Reduced Spi-B Levels Block pDCs, but Stimulate Myeloid and B Cell Differentiation In Vivo. To confirm the in vitro observations in an in vivo setting, we made use of a humanized SCID mouse model (24). We modified this model in that we injected irradiated newborn RAG-$2^{-/-}$ $\gamma_c^{-/-}$ mice directly in the liver with $CD34^+$ $CD38^-$ progenitor cells (25, 26). In this model development of pDCs, B cells and myeloid cells are already observed within 5 wk after injection. To compensate for the experimental variations between individual mice and to take into account differences in engraft-

Table II. *Spi-B RNAi Impairs pDCs and Stimulates Myeloid and B Cell Differentiation In Vivo*

	Bone marrow $BDCA2^+$			Bone marrow $CD14^+$			Bone marrow $CD19^+$			Spleen $BDCA2^+$			Liver $BDCA2^+$		
Experiment no.	ΔNGFR	Spi-Bi	Ratio[a]	ΔNGFR	Spi-Bi	Ratio[a]	ΔNGFR	Spi-Bi	Ratio[a]	ΔNGFR	Spi-Bi	Ratio[a]	ΔNGFR	Spi-Bi	Ratio[a]
1	852	45	5	406	1,270	313	1,461	1,769	121	83	8	10	3,445	0	0
2	4,476	241	5	3,545	3,744	106	496	1,160	234	307	11	3	43	0	0
3	1,750	835	48	770	1,582	206	16	2,054	12,783	309	29	9	97	23	24

[a]×100%.

The numbers represent absolute numbers of output cells of each subpopulation derived from 1,000 transduced input progenitor cells. The ratios (×100%) are representative for the relative increase or decrease of the Spi-Bi/GFP^+ cells compared to the control $\Delta NGFR^+$ cells.

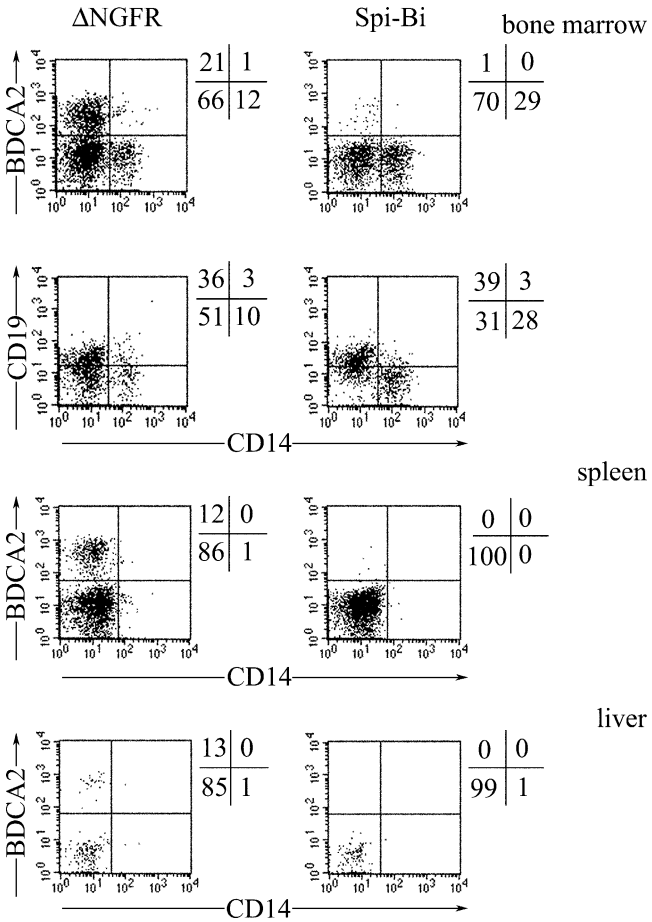

Figure 3. Spi-B knock down hampers pDCs, but not B and myeloid, development in vivo. 5–7-d-old irradiated neonatal RAG-$2^{-/-}$ $\gamma_c^{-/-}$ knockout mice were reconstituted with a mixture of Spi-Bi/GFP–transduced, control ΔNGFR–transduced, and nontransduced $CD34^+$ $CD38^-$ progenitor cells. Pictures show the transduced Spi-Bi/GFP^+ and control $\Delta NGFR^+$ human ($CD45^+$) cell populations present in the bone marrow, spleen, and liver 5 wk after injection: pDC ($BDCA2^+$), B cells ($CD19^+$) and myeloid cells ($CD14^+$). The data shown represents one of three experiments with comparable results.

ment of retrovirally transduced (cycling) cells versus nontransduced (mostly resting) $CD34^+$ $CD38^-$ cells, Spi-Bi/GFP-transduced cells were coinjected with $CD34^+$ $CD38^-$ cells transduced with a control construct expressing a signaling-incompetent mutant of the NGFR. At 5 wk after injection of both Spi-Bi/GFP and control ΔNGFR-transduced and nontransduced cells, we analyzed bone marrow, liver, spleen, thymus, and peripheral blood for the presence of human pDCs, B cells, and myeloid cells. Fig. 3 and Table II clearly show a strong reduction in the percentages and absolute cell numbers of $BDCA2^+$ pDCs in the Spi-Bi/GFP^+ population as compared with the control $\Delta NGFR^+$ cells in the bone marrow, spleen, and liver. Importantly, in the bone marrow we observed a higher percentage and absolute cell number of myeloid cells ($CD14^+$) and B cells ($CD19^+$) in the Spi-Bi/GFP^+ as compared with the control $\Delta NGFR^+$ population, indicating that development of $CD14^+$ and $CD19^+$ cells is stimulated by knocking down Spi-B.

Our findings clearly indicate that Spi-B is specifically required for development of human pDCs. These data add to observations that mice deficient for ICSBP/IRF-8 lack pDCs and $CD8\alpha^+$ DCs (13). Thus, both the ETS factor Spi-B and the IRF factor ICSBP/IRF-8 appear to be essential for pDC development. Interestingly, the ETS and IRF factors can cooperatively assemble on composite ETS-IRF DNA (EICE) elements, which were initially discovered in the immunoglobulin light chain enhancers but have later been found in promoters and enhancers of B lymphoid and myeloid genes (27). Similar to PU.1 and IRF-4, Spi-B and ICSBP/IRF-8 assemble in an ETS–IRF ternary complex of which the crystal structure was resolved recently (27). Given that both ICSBP/IRF-8 and Spi-B are required for pDC development, the structural data of Escalante et al. (27) make it very likely that Spi-B and ICSBP/IRF-8 cooperate in controlling pDC development.

Recent data have made clear that pDCs can develop both from $Flt3^+$ lymphoid as well as myeloid precursors (7, 8) and not solely from lymphoid precursors as we hypothesized earlier (5). More recently, Shigematsu et al. (28) reported that pDCs developing from myeloid precursors are phenotypically and functionally similar to those developing from lymphoid precursors. Interestingly, pDCs express pTα and have IgH D-J rearrangements regardless of their developmental origin. Importantly, both pDC developmental pathways result in expression of Spi-B (28). These findings strongly suggest that the pDC program can be induced by the interaction of Spi-B and ICSBP/IRF-8 in both lymphoid and myeloid precursor cells. It will be of interest to elucidate the mechanisms of induction and operation of this program.

Id proteins have also been implicated in the control of pDC development. We have demonstrated that overexpression of Id2 and Id3 strongly inhibit development of pDCs (5). Overexpression of DNA encoding the bHLH factors HEB and E2A into $CD34^+$ $CD38^-$ cells stimulated pDC development in a way comparable to Spi-B (unpublished data), which may suggest that these factors play a role in pDC development. The involvement of bHLH factors and Spi-B in pDC development raises the question whether these factors are collaborating in development of pDCs. This is possible, however, in contrast to Spi-B knock down, the forced expression of Id2 and Id3 strongly inhibits B cell development (29), which implies that either another yet to be identified bHLH factor cooperates with Spi-B in controlling pDC development or Spi-B collaborates with factors such as HEB, E12, and E47 in a cell context–dependent way.

The finding that Spi-B is specifically involved in development of pDCs suggests that it is a master gene for this cell type. This idea is supported by our previous findings that overexpression of Spi-B inhibits development of T cells, B cells, and NK cells (14), indicating that like with other master genes, expression of Spi-B in $CD34^+$ precursor cells is incompatible with alternative cell fates. Consistent with this notion, down-regulation of Spi-B stimulates development of "alternative" cell lineages such as B cells

and $CD11c^+$ $CD14^+$ myeloid cells. Based on the observation that overexpression of Spi-B impairs T cell development in vitro, it was expected that lowered levels of Spi-B would also stimulate T cell development. However, after injection of the mixture of Spi-Bi/GFP-transduced and control ΔNGFR–transduced $CD34^+$ progenitor cells in RAG-$2^{-/-}$ $\gamma_c^{-/-}$ mice, we observed high levels of control $\Delta NGFR^+$ cells, but not Spi-Bi/GFP^+ cells, in the thymus (not depicted), suggesting that Spi-B is either required for population of the thymus by precursor cells or Spi-B plays a crucial role in the early stages of development of T cells in the thymus. Experiments to investigate the effects of down-regulation of Spi-B on T cell development in vitro and in vivo are currently being performed in our lab.

This paper demonstrates the power of the RNAi approach for the study of human hematopoietic development. We have also demonstrated that the combination of this approach with the human SCID mouse model provides unique tools to unravel the roles of transcription factors and other gene products in human hematopoietic development in an in vivo setting.

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