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Enforced Expression of Spi-B Reverses T Lineage Commitment and Blocks β-Selection[1]

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The molecular changes that restrict multipotent murine thymocytes to the T cell lineage and render them responsive to Ag receptor signals remain poorly understood. In this study, we report our analysis of the role of the Ets transcription factor, Spi-B, in this process. Spi-B expression is acutely induced coincident with T cell lineage commitment at the $CD4^-CD8^-CD44^-CD25^+$ (DN3) stage of thymocyte development and is then down-regulated as thymocytes respond to pre-TCR signals and develop beyond the β-selection checkpoint to the $CD4^-CD8^-CD44^-CD25^-$ (DN4) stage. We found that dysregulation of Spi-B expression in DN3 thymocytes resulted in a dose-dependent perturbation of thymocyte development. Indeed, DN3 thymocytes expressing approximately five times the endogenous level of Spi-B were arrested at the β-selection checkpoint, due to impaired induction of Egr proteins, which are important molecular effectors of the β-selection checkpoint. T lineage-committed DN3 thymocytes expressing even higher levels of Spi-B were diverted to the dendritic cell lineage. Thus, we demonstrate that the prescribed modulation of Spi-B expression is important for T lineage commitment and differentiation beyond the β-selection checkpoint; and we provide insight into the mechanism underlying perturbation of development when that expression pattern is disrupted. ***The Journal of Immunology,*** **2005, 174: 6184–6194.**

T cell development is initiated upon colonization of the thymus by hemopoietic precursors, which then undertake a prescribed developmental program marked by changes in expression of the differentiation Ags, CD44, CD25, CD4, and CD8 (Fig. 1). The least mature of the thymocyte subsets, termed DN1[5] ($CD44^+CD25^-CD4^-CD8^-$), has the potential not only to give rise to T lymphocytes, but also $CD8^+$ thymic dendritic cells (DC), NK cells, and B lymphocytes, although the B lymphoid fate is blocked in the thymus by signals from the Notch pathway (1–3). Lineage potential becomes increasingly restricted during the ensuing stages, with the ability to give rise to DC remaining through the DN2 ($CD44^+CD25^+CD4^-CD8^-$) stage followed by irreversible commitment to the T cell lineage at the DN3 ($CD44^-CD25^+CD4^-CD8^-$) stage (1, 4). DN3 thymocytes do not manifest progenitor activity for other lineages under any conditions reported to date, including ectopic expression of the IL-2Rβ subunit, which is able to reveal latent myeloid potential in DN1 and DN2 thymocytes (4, 5). Although the mechanistic basis for progressive restriction to the T lineage remains poorly understood, recent evidence has revealed that Notch signaling is required (3, 6–8).

Development of committed T lineage cells beyond DN3 to the $CD4^+CD8^+$ (DP) stage is dependent upon generation of the TCR β subunit by V(D)J recombination. DN3 thymocytes that fail to productively rearrange the *TCRβ* locus are eliminated at the β-selection checkpoint, which stipulates that only thymocytes that maintain the translational reading frame of *TCRβ* will differentiate beyond DN3 to the DP stage (9–11). The signals promoting survival and differentiation of β-selected DN3 thymocytes are transduced by the pre-TCR complex, which comprises the TCRβ subunit in association with pre-Tα and the CD3 complex (12, 13). Pre-TCR signaling produces a number of developmental outcomes including rescue from apoptosis, entry into cell cycle, allelic exclusion at the *TCRβ* locus, and differentiation beyond DN3 to the DP stage (10, 14, 15). Among the vast array of changes in gene expression triggered by pre-TCR activation is the hallmark phenotypic change associated with β-selection, down-modulation of CD25 (16–19).

The use of gene-targeting and transgenic methodologies continue to provide insight into the molecular effectors of pre-TCR signaling and the β-selection differentiation program. Pre-TCR

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[5] Abbreviations used in this paper: DN1, $CD44^+CD25^-CD4^-CD8^-$; DN2, $CD44^+CD25^+CD4^-CD8^-$; DN3, $CD44^-CD25^+CD4^-CD8^-$; DN4, $CD44^-CD25^-CD4^-CD8^-$; DP, $CD4^+CD8^+$; DC, dendritic cell; Egr, early growth response; TAC:CD3ε, human IL-2Rα exo and transmembrane domains fused to the cytoplasmic domain of CD3ε; IRES, internal ribosomal entry site; eGFP, enhanced GFP; CFP, cyan fluorescent protein; LZRS, LZRSpBMN-linker-IRES-eGFP; pMIC, p-MSCV-IRES-CFP; FTOC, fetal thymic organ culture; SCF, stem cell factor; SA, streptavidin; MUG, methyl-umbelliferyl-β-D-galactoside; MHC-II, MHC class II; IRF, IFN regulatory factor; Id3, inhibitor of DNA binding 3.

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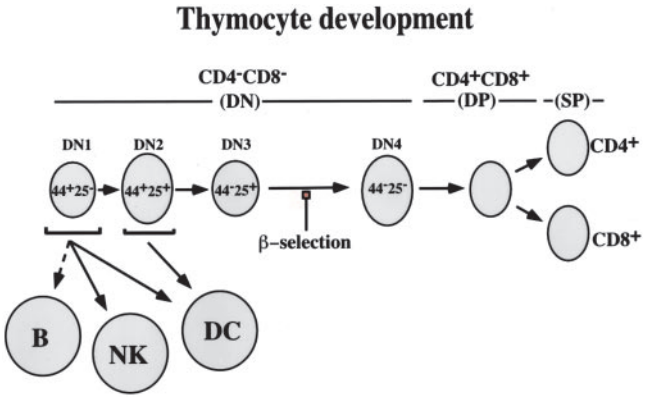

FIGURE 1. Schematic of thymocyte development. Stages of thymocyte development are indicated by differentiation Ags CD4, CD8, CD25, and CD44.

signaling depends heavily on the activities of the lck and ZAP-70 tyrosine kinases (20–23), the molecular adaptors Src homology 2 domain-containing leukocyte protein, 76 kDa, and linker for activation of T cells (24–26), as well as a number of DNA binding proteins including high mobility group box factors, Tcf-1 and Lef-1 and their coactivator β-catenin (27, 28), E box family basic helix-loop-helix proteins (29, 30), c-myb (31), and the early growth response (Egr) family of zinc finger transcription factors (32–34). Despite the identification of a number of effectors of pre-TCR signaling, our understanding of how the β-selection differentiation program is elaborated in response to pre-TCR signals remains incomplete. This is particularly true for the DNA binding proteins responsible for the changes in gene expression underlying differentiation.

The Ets transcription factors are a large family (~30) defined by a winged helix-turn-helix DNA binding domain, which recognizes the consensus GGAA/T motif (35). Ets proteins are critical regulators of lineage commitment during hemopoiesis (reviewed in Ref. 36); however, with the exception of the founding member, Ets-1, their role in thymocyte development remains poorly understood (37). Of the nine Ets family members expressed in developing thymocytes, three have been reported to be dynamically regulated during the early phases of thymocyte development (Erg, PU.1, and Spi-B), suggesting that they may play important roles (38). The Ets DNA binding domain of Erg is similar to the founding Ets family member, Ets-1; however, although the Ets domains of PU.1 and Spi-B are ~70% identical with each other, they are not as closely related to other Ets family members, and bind to a noncanonical DNA sequence motif (39). Gene-targeting of PU.1 arrests thymocyte development at a stage before commitment to the T cell lineage (40, 41). PU.1-deficient thymocytes that do survive and commit to the T cell lineage appear to develop normally thereafter (40). In Spi-B-deficient (Spi-$B^{-/-}$) mice, no defect in adult thymocytes was noted, although signaling through the surface Ig of mature B lymphocytes is impaired (42). Overexpression of both PU.1 and Spi-B has been shown to divert heterogeneous fetal thymocyte populations away from the T cell lineage, suggesting that the down-modulation of PU.1 that occurs before T lineage commitment is important for that commitment process (43, 44). Nevertheless, it is not clear whether overexpression of PU.1 or Spi-B after commitment to the T lineage might perturb T lineage commitment and traversal of the β-selection checkpoint. This is of particular importance for Spi-B, because unlike PU.1, whose expression is silenced during T-lineage commitment, Spi-B expression is acutely induced at the DN3 stage coincident with commitment to the T cell lineage and is then down-modulated during β-selection (38).

This manuscript focuses on the function of Spi-B in development of thymocytes that have already committed to the T cell lineage (i.e., DN3 cells). Gain-of-function analysis revealed that enforced expression of Spi-B in DN3 thymocytes results in a dose-dependent perturbation of development. Expression of Spi-B at high levels (~30 times endogenous) diverts even T lineage-committed DN3 to the DC lineage, whereas expression of lower levels of Spi-B (~5 times endogenous) arrests them at the β-selection checkpoint. Arrest at the β-selection checkpoint appears to result from impaired induction of the Egr family of transcription, because enforced expression of Egr proteins is able to overcome the arrest. Loss-of-function analysis revealed that Spi-B deficiency caused a delay in development of fetal DN thymocytes beyond the β-selection checkpoint to the DP stage. Thus, this report demonstrates that Spi-B function is necessary for efficient development of fetal DN thymocytes beyond the β-selection checkpoint to the DP stage; however, if Spi-B is too highly expressed or is not down-regulated normally, it interferes with differentiation beyond the β-selection checkpoint.

Materials and Methods

Mice

C57BL/6 and Spi-B-deficient (Spi-$B^{-/-}$) mice were maintained under specific pathogen-free conditions in the American Association of Laboratory Animal Care-accredited animal colony of the Fox Chase Cancer Center and were handled in compliance with guidelines established by the Institutional Animal Care and Use Committee (42). Compound PU.1/Spi-B-deficient animals were generated in the American Association of Laboratory Animal Care-accredited laboratory animal facility at State University of New York, Buffalo.

Cell lines

The Scid.adh-TAC:CD3ϵ thymic lymphoma was produced by retroviral transduction with the human IL-2Rα exo and transmembrane domains fused to the cytoplasmic domain of CD3ϵ (TAC:CD3ϵ) signaling chimera, as described (45). The Phoenix-E retroviral packaging line was provided by Dr. G. Nolan (Stanford University, Stanford, CA). All cell lines were maintained in Iscove's medium supplemented as described (32).

Retrovirus production

Full-length murine cDNAs for Spi-B, PU.1, and Erg were cloned from DN3 thymocytes by PCR and, after sequence verification, were subcloned into the retroviral vector LZRSpBMN-linker-internal ribosomal entry site (IRES)-enhanced GFP (eGFP) (LZRS). cDNAs encoding Egr1, -2, and -3 were subcloned into the retroviral vector p-MSCV-IRES-cyan fluorescent protein (CFP) (pMIC) (a gift from Dr. D. Vignali, St. Jude's, Nashville, TN). Phoenix-E retroviral packaging cells were transfected with retroviral vectors using the calcium phosphate transfection method as described (46). Transfection efficiencies were evaluated by determining the percentage of eGFP- or CFP-positive Phoenix-E cells using flow cytometry (FACSVantage SE; BD Biosciences).

Retroviral transduction and culture

Scid.adh-TAC:CD3ϵ cells were transduced with retroviral supernatant treated with 8 μg/ml polybrene and isolated by flow cytometry based on the retrovirally encoded fluorescent indicator protein, as previously described (32). Retroviral transduction and fetal thymic organ culture (FTOC) were performed as described previously (32). Briefly, single-cell suspensions of day 14 fetal C57BL/6 thymocytes were transduced with Lipofectamine-treated (20 μg/ml; Invitrogen Life Technologies) viral supernatant, incubated overnight at 37°C, seeded onto deoxyguanosine-depleted day 15 C57BL/6 host lobes (30,000/lobe) in Terasaki plate hanging drop cultures, and then incubated for 2 days at 37°C before analysis. For DC progenitor cultures, DN2 and DN3 thymocytes purified by cell sorting were virally transduced and seeded into deoxyguanosine-treated lobes as above or cultured in suspension at 25,000 cells per 100 μl in 96-well flat-bottom plates for 5 days in medium supplemented with cytokines as described (4): 1 ng/ml TNF-α, 0.2 ng/ml IL-1β, 400 ng/ml IL-3, 100 ng/ml

IL-7, 10 ng/ml stem cell factor (SCF), and 100 ng/ml Flt3 ligand (R&D Systems).

Flow cytometry

Single-cell suspensions were preincubated for 10 min at 4°C with anti-FcRγII/III (clone 2.4G2) Ab to block nonspecific FcR binding and subsequently stained with the indicated Ab for 30 min at 4°C. After washing in FACS buffer (1% BSA/HBSS containing 0.02% sodium azide), cells stained with biotinylated primary Ab were incubated for 10 min with the indicated fluorochrome-conjugated avidin secondary reagent and washed before analysis using a FACSVantage SE and FlowJo software (Tree Star). Dead cells were excluded using the vital dye propidium iodide. Biotinylated, FITC-, PE-, allophycocyanin-, PE-Cy7-, or CyChrome-conjugated Ab specific for CD4 (clone GK1.5 or RM4-5), CD8α (clone 53-6.7), CD11b (M1/70), CD11c (HL3), CD25 (IL-2Rα; clone PC61), CD27 (clone LG.3A10), CD28 (clone 37.51), CD44 (clone IM7), CD80 (B7.1; clone 16-10A1), CD86 (B7.2; clone GL1), MHC class II (MHC-II) (I-A/I-E; clone M5/114.15.2), TCRβ (clone H57-597), and TCR$\gamma\delta$ (clone GL3) were obtained from BD Pharmingen. Streptavidin (SA)-allophycocyanin, SA-CyChrome, and avidin-Texas Red were obtained from BD Pharmingen, whereas TCR$\gamma\delta$ (clone GL3)-Tricolor and SA-PE-Cy7 were obtained from Caltag.

For intracellular TCRβ staining, cells were surface stained as described above and subsequently washed with 4°C HBSS, fixed for 15 min at room temperature with 0.5% *p*-formaldehyde/PBS, and quenched with 50 mM ammonium chloride/PBS. Fixed cells were permeabilized with saponin (0.5% saponin/5% FCS/HBSS) on ice for 30 min and stained for 30 min on ice with anti-*TCRβ* (H57-597). After two washes in permeabilization buffer, cells were analyzed as above on the FACSVantage SE.

Ag presentation assay

T cell hybridomas specific for the S1 and S3 epitopes of influenza virus PR8 hemagglutinin were used in Ag presentation assays that have been described in detail elsewhere (47, 48). Scid.adh cells were incubated with synthetic peptides corresponding to the S1 and S3 epitopes of influenza virus PR8 HA (S1, aa 107–119, SVSSFERFEIFPK; and S3, aa 302–313, CPKYVRSAKLRM; ResGen; Invitrogen Life Technologies) at 10 μg/ml and cocultured with T cell hybridomas for 16–18 h. T cell responses were measured using the fluorogenic substrate methyl-umbelliferyl-β-D-galactoside (MUG; purchased from Sigma-Aldrich) according to the method described by Sanderson and Shastri (48).

Cell stimulation

Scid.adh-TAC:CD3ϵ cells were plated in complete Iscove's medium (2×10^5/well) in 24-well tissue culture plates precoated with 2.5 μg/well anti-TAC Ab. The anti-TAC mAb-producing hybridoma hd245/332 was obtained from the American Type Culture Collection with the permission of Dr. T. Waldman (National Institutes of Health, Bethesda, MD). After incubation for 24 h at 37°C, cells were analyzed by FACS and, where indicated, sorted for eGFP and CD11c expression.

Semiquantitative RT-PCR and Southern blot analysis

Total RNA was isolated using the RNeasy RNA purification system (Qiagen) and RT-PCR Southern blots were performed as described (32). Titrated amounts of cDNA were amplified by PCR. β-Actin, TCR-Cα, Egr1, Egr2, and Egr3 primers were described previously (45, 49). Additional primers used are as follows: Erg, 5′-TGAAGACCAGCGTCCTCAGT TAG-3′ and 5′-GCAATCCCGTGGAAGTCAAAC-3′; inhibitor of DNA binding 3 (Id3), 5′-ATGAAGGCGCTGAGCCC-3′ and 5′-GTGGC AAAAGCTCCTCTTG-3′; PU.1, 5′-TGACTACTACTCCTTCGTGGG CAG-3′ and 5′-TTCTCCATCAGACACCTCCAGG-3′; Spi-B, 5′-CAT GCTTGCTCTGGAGGCTGCACA-3′ and5′-AGCAGGATCGAAGGCTT CATAGGG-3′. After hybridization and washing, the radioactive signal was quantified using a Fuji phosphor imager and Fuji MacBas version 2.2 software (Fuji Photo Film) and normalized to β-actin.

Immunofluorescence

Day 18.5 embryonic thymi were isolated and embedded in Tissue-Tek OCT (Sakura Finetek). Six-micrometer sections were fixed with 95% ethanol, blocked with 5% normal goat serum, and stained with biotin-labeled anti-CD11c (clone HL3; BD Pharmingen) for 30 min at room temperature. Bound Ab was visualized with avidin-Alexa-488 (Molecular Probes). After mounting using Fluoromount-G (Southern Biotechnology), samples were viewed using a Nikon Optiphot epifluorescence microscope equipped with a Quad-Fluor (EF-1) four-cube filter holder loaded with appropriate filters. Images were recorded using the Qimaging Retiga-1300 cooled charge-coupled device camera and processed with Openlab software (Improvision).

Results

Dynamic expression of Ets transcription factors during fetal thymocyte development

Although the expression of PU.1, Erg, and Spi-B has been found to be dynamically regulated during development of adult thymocytes, this had not been investigated during fetal development. We found that the expression pattern of PU.1, Erg, and Spi-B within DN subsets 1–4 (DN1, $CD44^+CD25^-$; DN2, $CD44^+CD25^+$; DN3, $CD44^-CD25^+$; DN4, $CD44^-CD25^-$) from day 14 fetal mice differed substantially (Fig. 2). PU.1 and Erg were expressed predominantly in the DN1 and DN2 subsets; however, Spi-B exhibited a reciprocal expression pattern. Spi-B was expressed at very low levels in DN1–2 but was strongly induced at the DN3 stage coincident with commitment to the T lineage and then down-regulated during development beyond the β-selection checkpoint to the DN4 stage (Fig. 2). The marked up-regulation of Spi-B coincident with commitment to the T cell lineage at the DN3 stage raised the possibility that Spi-B might play a predominant role during and after T cell lineage commitment. These results differ slightly from those reported previously following analysis of thymic subpopulations isolated from adult mutant mice in that we did not find Spi-B expression to be elevated in DN1 thymocytes, perhaps reflecting differences in expression patterns between fetal and adult thymic development (38).

Effect of Ets factor overexpression on thymocyte development in organ culture

Because Erg, PU.1, and Spi-B exhibited distinct patterns of expression, we asked whether disruption of their expression patterns would lead to distinct effects on thymocyte development. The Ets factors were retrovirally transduced into day 14 fetal C57BL/6 thymocytes using the LZRS vector, whose IRES linker enables the eGFP indicator both to identify infected cells and to estimate the expression level of the gene of interest. Transduced thymocytes were cultured in hanging drop culture with a T cell-depleted host lobe for 2 days, and then analyzed by flow cytometry using Ab reactive with CD44 and CD25. Interestingly, retroviral transduction of these three transcription factors led to distinct alterations in

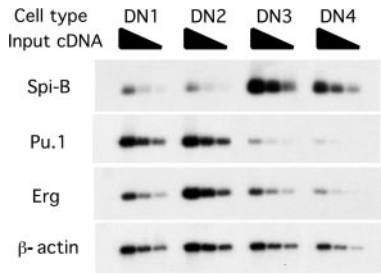

FIGURE 2. Spi-B mRNA levels are down-modulated during development as DN3 thymocytes develop beyond the β-selection checkpoint. Levels of mRNA encoding Erg, PU.1, and Spi-B were assessed by RT-PCR Southern blotting on the indicated DN subsets that were isolated by flow cytometry from day 15 C57BL/6 embryos based on their CD44 and CD25 expression profiles. CD4, CD8, $\gamma\delta$ TCR-expressing cells were excluded. Total RNA was reverse transcribed, and then template cDNA was serially diluted (neat, 1:3, and 1:6) before PCR amplification. Amplified fragments were then resolved on agarose gels, transferred to membranes, and visualized by hybridization with the appropriate ^{32}P-labeled probe. Samples that had not been reverse transcribed were included to control for genomic DNA contamination.

thymocyte development. Enforced expression of Erg led to an accumulation of cells at the DN3 stage, suggesting that its down-modulation during β-selection is important for traversal of that checkpoint (Fig. 3*A*). In contrast, PU.1 transduction caused an arrest of development at the DN1 and DN2 stages, consistent with a previous report (43). Spi-B transduction resulted in both a modest accumulation of cells at the DN1 and -2 stages and a block at the DN3 stage (Fig. 3A, *right*). Because thymocytes at the DN1 and -2 stages are not yet committed to the T cell lineage (see Fig. 1), we assessed whether PU.1 and Spi-B overexpression arrested development, diverted thymocytes to a distinct lineage fate, or both. PU.1 expression up-regulated the DC marker CD11c on essentially all transduced thymocytes within 2 days of hanging drop culture, indicating diversion to the DC lineage (Fig. 3*B*, *middle*). This is consistent with a previous report indicating that PU.1 transduction promoted thymocytes to adopt a myeloid fate in long-term cultures (43). Spi-B transduction also up-regulated CD11c on a substantial fraction of transduced cells (Fig. 3*B*, *right*). These $CD11c^+$ cells exhibited other characteristics of the DC lineage, including expression of MHC-II Ags, CD11b, CD80, CD86, and a broader side-scatter profile (data not shown). Importantly, whereas enforced expression of PU.1 and Spi-B induced DC lineage commitment, this was not observed in Erg-transduced thymocytes, which remained CD11c negative and arrested at the DN3 stage. Unlike PU.1-transduced cells, the Spi-B-transduced population contained a substantial number of cells that had not up-regulated CD11c. The bimodal CD11c expression pattern of Spi-B-transduced cells appeared to correlate with the Spi-B expression level, because the $CD11c^+$ fraction of Spi-B-transduced cells was approximately five to six times brighter for eGFP, suggesting that they expressed a higher level of Spi-B than those cells that remained CD11c negative and were arrested at the β-selection checkpoint at DN3 (Fig. 3*C*). To gain insight into the extent of overexpression relative to endogenous Spi-B levels, day 14 fetal thymocytes were transduced with LZRS-Spi-B, and after 24 h, Spi-B expression was assessed on the $eGFP^+$ population. RT-PCR Southern blotting revealed that retroviral transduction of Spi-B resulted in ~25-fold overexpression relative to endogenous Spi-B levels (Fig. 3*D*). Considering the proportions of the $CD11c^+$ and $CD11c^-$ subpopulations and their relative GFP levels, we estimate that the $CD11c^+$ LZRS-Spi-B-transduced cells express ~30 times the endogenous Spi-B level, whereas the $CD11c^-$ cells arrested at the β-selection checkpoint express approximately five times the endogenous Spi-B level. Therefore, whereas enforced expression of PU.1 diverted essentially all developing thymocytes to the DC lineage and enforced expression of Erg blocked development at the β-selection checkpoint, Spi-B transduction produced an apparent dose-dependent perturbation of development, with high expressers adopting the DC fate and lower expressers arresting at the β-selection checkpoint.

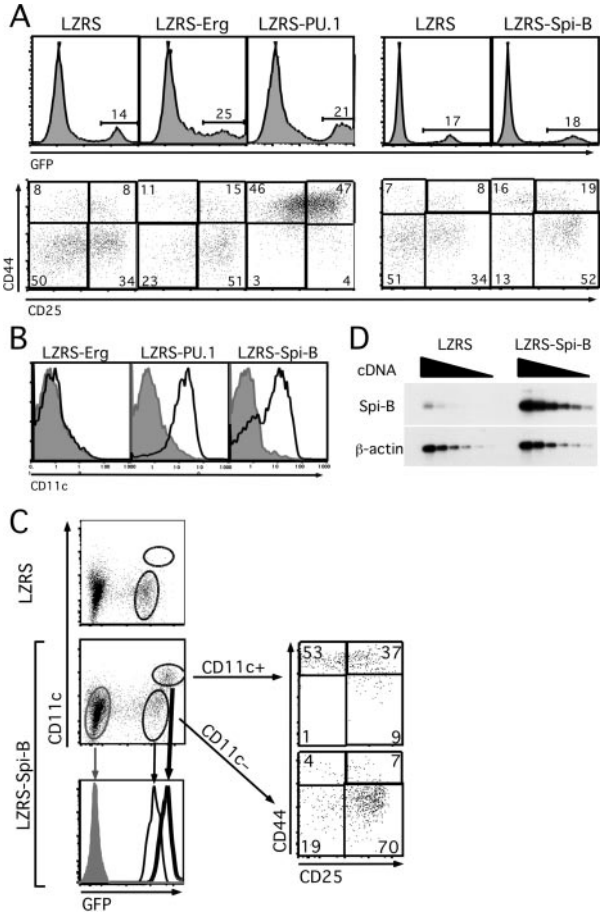

FIGURE 3. Enforced expression of Spi-B results in a dose-dependent perturbation of fetal thymocyte development. *A–C*, Day 14 fetal C57BL/6 thymocytes that had been retrovirally transduced with the indicated viral construct were seeded into alymphoid thymic lobes for 2 days in hanging drop culture before flow-cytometric analysis. Results are representative of at least three experiments. Cell recoveries for all cultures were comparable. *A*, Thymocyte suspensions were stained with Ab reactive to CD44 and CD25. *Upper panels* contain histograms indicating the percentage of GFP^+ cells resulting from transduction with each viral construct. CD44/CD25 profiles of the corresponding GFP^+ subsets are found in the *lower panels*. *B*, Thymocyte suspensions treated as in *A* were additionally stained with anti-CD11c. The CD11c expression profile of GFP^+ cells from control-transduced thymocytes (LZRS, filled gray) was compared with that of cultures transduced with the indicated gene of interest (black line). *C*, The CD44/CD25 profiles of electronically gated $CD11c^+$ and $CD11c^-$ LZRS-Spi-B-transduced cells (from *B*) as well as their relative GFP expression levels (thick and thin black lines, respectively) were compared. *D*, The extent of Spi-B overexpression achieved by retroviral transduction was assessed by performing RT-PCR Southern blots on total RNA isolated from the GFP^+ populations of day 14 fetal thymocytes 24 h after retroviral transduction with LZRS or LZRS-Spi-B retrovirus. Samples represent a 3-fold dilution series of input cDNA. Reverse-transcribed control PCR were performed for each sample.

Stage dependence of the effects of Spi-B overexpression on thymocyte development

Because the above experiments were performed on a thymocyte population with mixed lineage potential, it was unclear whether the observed perturbation of development resulted from dysregulation of Ets proteins before or after commitment to the T lineage. To address this possibility, the effects of Spi-B overexpression on thymocyte development were re-evaluated on purified thymocyte subpopulations. DN2 thymocytes, which have DC precursor potential, and DN3 thymocytes, which are committed to the T cell lineage and are devoid of DC precursor potential, were isolated by cell sorting, retrovirally transduced, and analyzed as in Fig. 3 (Fig. 4*A*) (4). Although most DN2 thymocytes transduced with control vector differentiated to the DN3 stage ($CD44^-CD25^+$), those transduced with Spi-B were arrested at the DN1 ($CD44^+CD25^-$) and -2 ($CD44^+CD25^+$) stage and up-regulated CD11c (Fig. 4*B*). Importantly, the development of purified DN3 thymocytes was also perturbed by Spi-B transduction. Spi-B-transduced DN3 were either arrested at the DN3 stage or re-expressed CD44, thereby reverting to a DN2 phenotype (Fig. 4*B*). Moreover, almost half of

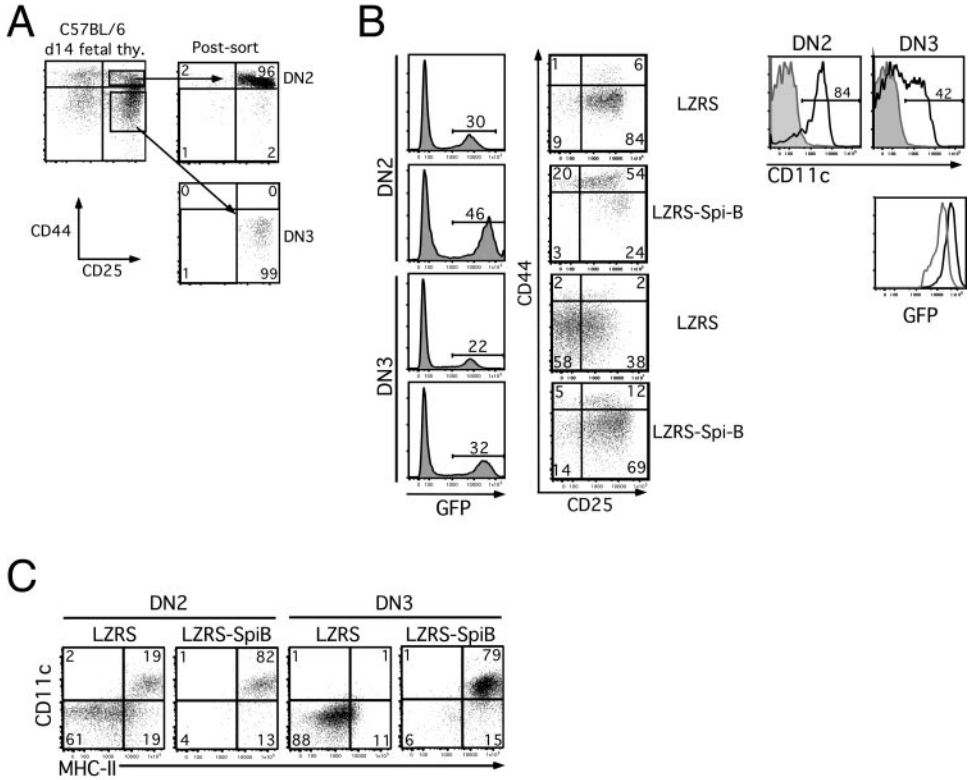

FIGURE 4. Transduction of DN3 thymocytes with Spi-B results in a dose-dependent perturbation of thymocyte development. *A*, DN2 thymocytes (the stage before T lineage commitment) and DN3 thymocytes (the stage at which thymocytes are committed to the T cell lineage) were isolated from day 14 (d14) C57BL/6 fetal thymocytes by flow cytometry and then reanalyzed to assess purity immediately after isolation. *B*, Purified DN2 and DN3 thymocytes were retrovirally transduced with either LZRS or LZRS-Spi-B, seeded into alymphoid thymic lobes for 2 days in hanging drop culture, and then analyzed by flow cytometry using the indicated Ab as in Fig. 3. The fraction of GFP^+ cells is indicated in the shaded single-color histograms on the *left*. CD44/CD25 and CD11c expression profiles of electronically gated GFP^+ populations from LZRS-transduced thymocytes were compared with those transduced with LZRS-Spi-B. In the *right panels*, CD11c staining on LZRS-transduced cells is indicated by filled gray profiles and that of LZRS-Spi-B-transduced cells by a black line. Comparison of the GFP levels of electronically gated $CD11c^+$ (thick black line) and $CD11c^-$ (thin black line) LZRS-Spi-B-transduced DN3 thymocytes revealed that the $CD11c^+$ cells expressed higher GFP levels (*lower right*). *C*, Purified DN2 and DN3 infected as in *B* were cultured in suspension for 6 days in a mixture of cytokines (TNF-α, IL-1β, IL-3, IL-7, SCF, and Flt3 ligand) demonstrated previously to induce uncommitted thymic precursors to adopt the DC lineage. Development was assessed by flow cytometry with the indicated Ab. Electronically gated GFP^+ cells are shown.

Spi-B-transduced DN3 cells up-regulated CD11c expression, consistent with diversion of these cells to the DC lineage. The Spi-B-transduced DN3 cells that had up-regulated CD11c also exhibited ~5-fold greater GFP expression (Fig. 4*B*, *lower right*). The ability of Spi-B transduction to divert DN3 thymocytes to the DC lineage was surprising given that DN3 thymocytes are T lineage committed and were previously thought to be incapable of adopting the DC lineage under any circumstances, including culture in the presence of a battery of cytokines capable of inducing DN2 thymocytes to adopt the DC lineage (Fig. 4*C*) (4). Indeed, whereas DN2 thymocytes were induced to up-regulate CD11c and MHC-II and adopt the DC fate in the presence of these cytokines (TNF-α, IL-1β, IL-3, IL-7, SCF, and Flt3 ligand), the cytokines were only capable of diverting to the DC lineage those DN3 cells that had been transduced with Spi-B (Fig. 4*C*). Taken together, these data demonstrate that the dose-dependent ability of Spi-B to divert thymocytes to the DC lineage is evident even in purified DN3 thymocytes.

Diversion of a T lymphoma to the DC lineage by Spi-B overexpression

To further examine the ability of enforced expression of Spi-B to divert T lineage cells to the DC lineage, we used the Scid.adh thymic lymphoma, which resembles DN3 thymocytes both phenotypically and in its ability to undergo a differentiation process that closely resembles that of a normal thymocyte undergoing β-selection in vivo (45). Indeed, as was observed with purified DN3 thymocytes, we found that transduction with LZRS-Spi-B induced a subpopulation of Scid.adh cells to undergo morphological alterations that suggested diversion of this T lymphoma to the DC lineage (Fig. 5*A*). A subpopulation of Spi-B-transduced Scid.adh cells, but not control-transduced cells, attached to the culture dish and extruded projections reminiscent of DC (Fig. 5*A*). Flow-cytometric evaluation revealed that almost half of the Spi-B-transduced population induced expression of CD11c; and the $CD11c^+$ population also expressed other markers of the DC lineage, including CD11b, MHC-II, CD80, and CD86 (Fig. 5*B*; data not shown). Moreover, like normal DN3 in vivo, diversion to the DC lineage appeared to be dose dependent, with the DC like cells expressing ~5-fold more Spi-B than those cells remaining CD11c negative. In addition to their morphologic and phenotypic resemblance of DC, these Spi-B-transduced thymic lymphoma cells also acquired the ability to present Ag to and activate peptide-responsive T hybridoma cells (Fig. 5*C*). Only the $CD11c^+$ population acquired the ability to present peptide, because the $CD11c^-$ cells had no effect on activation of the T hybridoma even in the presence of specific peptide (Fig. 5*C*). Taken together with our experiments on normal thymocytes (Fig. 4), these data represent the first example of committed T lineage cells being diverted to DC lineage.

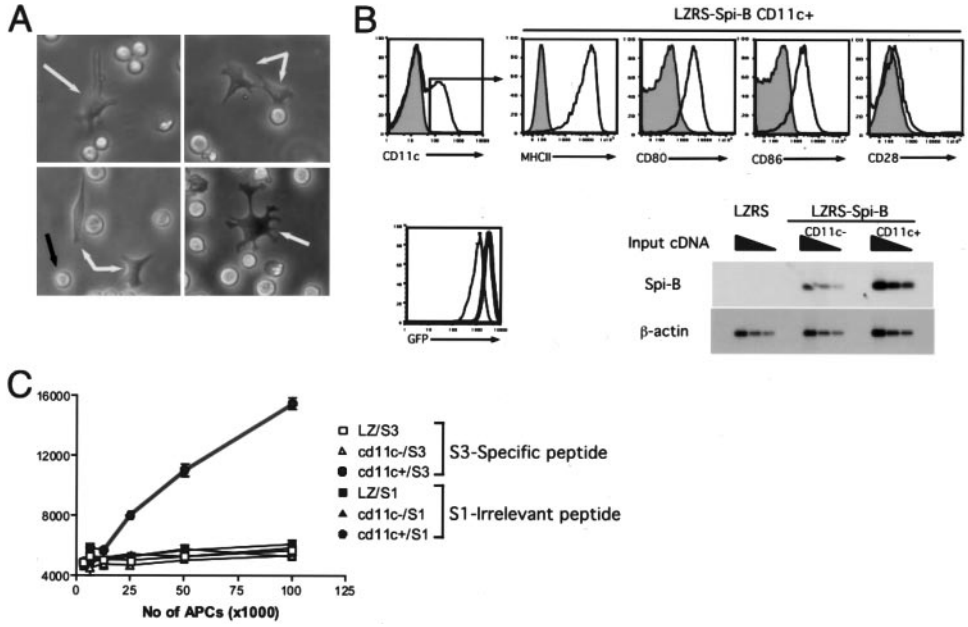

FIGURE 5. Enforced expression of Spi-B induces a DN3-like T cell line to exhibit DC characteristics. *A*, Light microscopy of Scid.adh cells (normal morphology; black arrow) 10 days after infection with LZRS-Spi-B revealed that a subpopulation of Scid.adh cells had adhered to the culture dish and elaborated membrane projections (white arrows). *B*, Scid.adh cells transduced with LZRS or LZRS-Spi-B were analyzed by flow cytometry for expression of markers expressed by DC: CD11c, MHC-II, CD80, and CD86. Expression of CD11c on LZRS (filled gray)- and LZRS-Spi-B (black)-transduced cells is compared in the histogram in the *upper left.* Histograms illustrating expression of MHC-II, CD28, CD80, and CD86 on electronically gated $CD11c^+$ LZRS-Spi-B-transduced cells are found in the *upper right*. The *bottom-left panel* depicts the GFP levels of $CD11c^-$ (thin black line) and $CD11c^+$ (thick black line) LZRS-Spi-B-transduced Scid.adh cells. RT-PCR Southern blots were performed on Scid.adh cells transduced with LZRS and on the $CD11c^-$ and $CD11c^+$ subpopulations of LZRS-Spi-B-transduced Scid.adh cells to assess the difference in Spi-B expression level (*bottom-right panel*). Input cDNA was diluted, amplified, and blotted as in Fig. 2. *C*, LZRS-transduced, LZRS-Spi-B-transduced $CD11c^-$, and LZRS-Spi-B-transduced $CD11c^+$ Scid.adh cells were loaded with peptides (S1 or S3) and then incubated with an S3-reactive T cell hybridoma. Activation of the hybridoma was assessed using a fluorogenic β-galactosidase substrate (MUG). MUG is cleaved by the β-galactosidase indicator enzyme that is induced upon hybridoma activation. The data are representative of three experiments performed.

DC development in Spi-B$^{-/-}$ mice

The ability of Spi-B overexpression to divert thymocytes to the DC lineage prompted us to investigate whether Spi-B deficiency might impair the development of thymic DC. However, flow-cytometric analysis revealed no significant difference in the frequency of $CD11c^+$ cells in the thymi of adult C57BL/6 and Spi-$B^{-/-}$ mice (0.20 ± 0.03 and $0.24 \pm 0.05\%$, respectively). Likewise, immunohistochemical analysis of fetal thymic lobes revealed no obvious difference in the number of $CD11c^+$ cells in sections of day 14 fetal thymic lobes cultured in FTOC for 1, 3, or 5 days (data not shown). Because Spi-B and PU.1 have been shown to exhibit functional redundancy in some developmental contexts (50, 51), we reasoned that the absence of a dramatic disruption of thymic DC development in Spi-$B^{-/-}$ mice may result from functional redundancy with PU.1, because one PU.1-deficient mouse line has been reported to develop thymic DC (52). Consequently, we analyzed thymi from the PU.1-deficient mouse line mentioned above, from Spi-B-deficient mice, and from mice deficient for both PU.1 and Spi-B. Immunofluorescent staining with anti-CD11c was performed to identify DC in frozen sections from fetal day 18.5 mice, because PU.1 deficiency is embryonic lethal (Fig. 6). This analysis revealed that DC were present in the thymi from mice that express both PU.1 and Spi-B and in mice singly deficient for Spi-B; however, DC were not detected in PU.1-deficient mice. This contrasts with previously reported analysis of this PU.1-deficient mouse line (52), but agrees well with analysis of the other PU.1-deficient mouse line in which development of both myeloid and lymphoid DC was reported to be blocked (53). As expected, DC were also absent from mice lacking both PU.1 and Spi-B. These data suggest that Spi-B is unable to support development of thymic DC in the absence of PU.1.

Effect of Spi-B deficiency on thymocyte development

Because the expression of Spi-B is acutely induced in the DN3 thymocyte subset in which β-selection occurs, we used Spi-B-deficient mice to determine whether Spi-B was important for traversal of this checkpoint. Because no abnormalities in development or function of T cells had previously been reported in adult Spi-$B^{-/-}$ mice (42), we examined the kinetics of development in FTOC. In fact, we did observe a consistent delay in development of DN Spi-$B^{-/-}$ fetal thymocytes beyond the β-selection checkpoint to the DP stage, such that the absolute number of DP generated in Spi-B-deficient thymocytes was ~3-fold lower than that in wild-type thymocytes (Fig. 7*A*); however, this delay was minimized by day 5 of culture. The molecular basis for the delay in development is currently unclear but does not appear to result from interference with expression of *TCRβ* or the IL-7R (data not shown), both of which have been suggested as potential Spi-B targets because of overlapping target specificity with PU.1. Thus, Spi-B is necessary for optimal development beyond the β-selection checkpoint to the DP stage. The disruption of development is relatively modest, perhaps due to functional redundancy with PU.1, because PU.1 deficiency results in an incomplete block in thymocyte development (40). This possibility will be the focus of future studies.

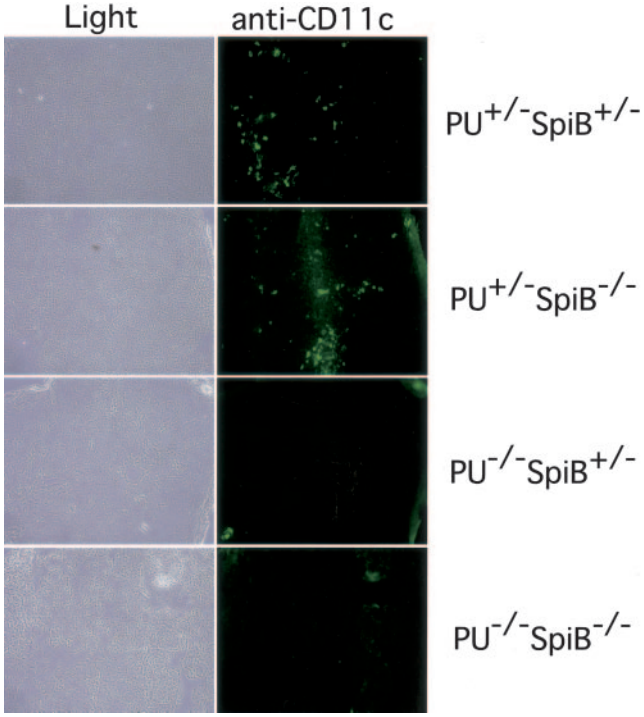

FIGURE 6. DC development is blocked by PU.1 deficiency. Day 18.5 fetal thymi from PU.1$^{+/-}$Spi-B$^{+/-}$, PU.1$^{+/-}$Spi-B$^{-/-}$, PU.1$^{-/-}$Spi-B$^{+/-}$, and PU.1$^{-/-}$Spi-B$^{-/-}$ mice were sectioned and stained with anti-CD11c Ab. Light and fluorescence micrographs are depicted.

Mechanism underlying blockade of β-selection by Spi-B overexpression

In considering the mechanism whereby enforced expression of Spi-B blocked β-selection, we reasoned that Spi-B might interfere either with pre-TCR expression or with the differentiation program elicited by pre-TCR signaling. Because PU.1 exhibits some functional redundancy with Spi-B and has previously been reported to suppress expression of the *RAG* genes (54), we asked whether Spi-B overexpression inhibited the production of *TCRβ* protein by performing intracellular *TCRβ* staining on Spi-B-transduced fetal thymocytes. In fact, Spi-B overexpression did not appear to interfere with generation of *TCRβ*, because the fraction of DN3 thymocytes that stained positive for intracellular *TCRβ* was somewhat higher (~29%) in LZRS-Spi-B-transduced cultures than in cultures transduced with control vector (~21%) (Fig. 7*B*).

Spi-B-mediated blockade of β-selection appeared to result from effects on pre-TCR function rather than expression; therefore, we used the Scid.adh model system described above to address this possibility. Following LZRS-Spi-B transduction, those Spi-B-expressing cells that remained CD11c$^-$ 4–5 days after infection were isolated by flow cytometry. To determine whether Spi-B expression interfered with Scid.adh differentiation as it did with β-selection of normal thymocytes in vivo, Spi-B-expressing Scid.adh cells were stimulated using the TAC:CD3ϵ signaling chimera. The TAC:CD3ϵ signaling chimera has been shown to mimic pre-TCR signaling in vivo (55). Moreover, we have previously shown that TAC:CD3ϵ signaling induced Scid.adh cells to undergo a program of differentiation that closely resembles that of normal thymocytes undergoing β-selection in vivo (45). Control (LZRS)- and Spi-B-expressing Scid.adh cells were stimulated through TAC:CD3ϵ by Ab cross-linking, and their differentiation was assessed by flow cytometry after 24 h. Importantly, the CD25 down-modulation that is characteristic of β-selection was almost completely abrogated in Spi-B-transduced cells (Fig. 8*A*). Likewise, Spi-B also interfered with induction of CD27 and CD28 (Fig. 8*A*), and with the up-regulation of TCR-Cα transcripts that normally accompanies pre-TCR signaling in vivo (*B*) (18, 56). These data demonstrate that Spi-B transduction interferes with execution of the Scid.adh differentiation program.

We and others have demonstrated that the Egr family of Zn^{2+} finger transcription factors plays an important role in traversal of the β-selection checkpoint (32–34). To determine whether induction of the Egr proteins were perturbed by Spi-B overexpression, we performed RT-PCR Southern blots on TAC:CD3ϵ stimulated Scid.adh cells transduced with vector alone (LZRS) or with Spi-B (LZRS-Spi-B). TAC:CD3ϵ stimulation induced expression of all three Egr proteins expressed in developing thymocytes (Egr1, -2, and -3) (Fig. 8*B*, *left*) (32). Importantly, although induction of Egr1 appeared normal in Spi-B-expressing cells, induction of Egr2 and Egr3 was impaired, as was the induction of the Egr protein target, Id3 (57). Although we have previously determined that induction of the Egr proteins is sufficient for traversal of the β-selection checkpoint, it should be noted that ablation of Egr3 alone partially impairs development beyond the β-selection checkpoint (34). Moreover, Egr2 and Egr3 are much more potent inducers of the helix-loop-helix factor, Id3, than is Egr1 (data not shown) (32). Id3 expression is normally induced during β-selection in vivo and acts to inhibit the function of E proteins (29, 58). Elimination of E protein activity is sufficient to enable thymocytes to traverse the β-selection checkpoint even in the absence of pre-TCR signals (29). Taken together, these data demonstrate that Spi-B overexpression inhibits induction of two important types of molecular effectors of the β-selection, Egr proteins and one of their downstream targets, Id3.

If interference with induction of Egr2 and Egr3 is mechanistically linked to the ability of Spi-B to impair β-selection, then enforced expression of Egr2 and/or Egr3 should restore Scid.adh differentiation. To test this possibility, control- and Spi-B-expressing Scid.adh cells were retrovirally transduced with Egr family members encoded by the pMIC vector, whose IRES links expression of the gene of interest to CFP, which is distinguishable from eGFP. We have previously demonstrated that enforced expression of Egr proteins can induce differentiation of normal thymocytes beyond the β-selection checkpoint and Scid.adh differentiation in vitro, even in the absence of pre-TCR signals (32). In accord with our previous results, gating on the CFP$^+$ population revealed that retroviral transduction with all three Egr family members expressed in developing thymocytes caused CD25 down-modulation in control, LZRS-transduced cells. Importantly, transduction of Spi-B-expressing Scid.adh cells revealed selectivity in the ability of Egr family members to overcome the block in differentiation. Indeed, enforced expression of Egr1, whose induction was unaffected by Spi-B overexpression, did not effectively induce CD25 down-modulation in Spi-B-expressing Scid.adh cells. In contrast, both Egr2 and Egr3, whose induction was blunted by Spi-B overexpression, were able to effectively induce CD25 down-modulation in Spi-B-transduced Scid.adh cells. These observations suggest that Spi-B is blocking differentiation of Scid.adh by inhibiting induction of Egr2 and Egr3, providing a mechanistic explanation for blockade of β-selection by enforced expression of Spi-B.

Discussion

We report here that the Ets factor Spi-B is acutely induced coincident with T lineage commitment in DN3 stage thymocytes and is then down-modulated during progression beyond the β-selection checkpoint. Deviation from the normal Spi-B expression pattern causes dose-dependent perturbations in thymocyte development.

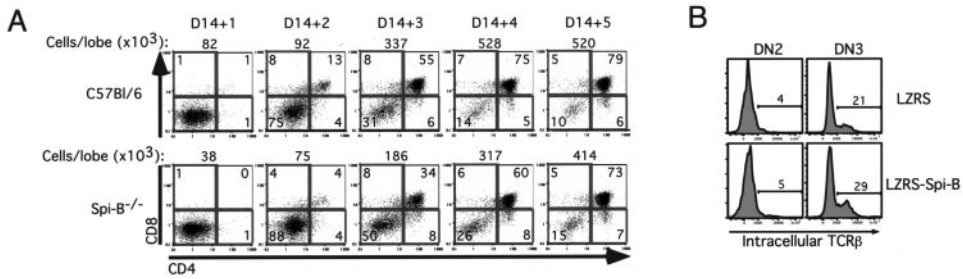

FIGURE 7. The impairment of thymocyte development associated with Spi-B deficiency or overexpression does not result from interference with *TCRβ* expression. *A*, FTOC were performed using day 14 (D14) fetal thymic lobes from Spi-B-deficient and C57BL/6 mice. Development was assessed daily by flow cytometry with the indicated Ab beginning at day 14 + 1. The cellularity is indicated above the histogram. This analysis is representative of four experiments performed. *B*, Day 14 fetal thymocytes transduced with LZRS or LZRS-Spi-B were analyzed after a 2-day hanging drop culture for the presence of intracellular *TCRβ* using flow cytometry. The histograms depict intracellular *TCRβ* staining on electronically gated DN2 and DN3 thymocytes transduced with either LZRS or LZRS-Spi-B.

Indeed, enforced expression of Spi-B by retroviral transduction causes those cells expressing approximately five times endogenous levels to arrest at the DN3 stage, supporting the hypothesis that down-modulation of Spi-B is important for execution of the β-selection differentiation program. Enforced expression of Spi-B appears to block β-selection by interfering with induction of the Zn^{2+} finger transcription factors, Egr2 and Egr3, which we have previously shown to be important effectors of the β-selection differentiation program (32, 34). Interestingly, those thymic precursors expressing higher levels of Spi-B (~30 times endogenous levels) are diverted to the DC lineage. It is noteworthy that high-level Spi-B expression was able to divert to the DC lineage purified DN3 thymocytes, which were previously thought to be irreversibly committed to the T cell lineage (4). These findings underscore the importance of tight control of Spi-B expression in order for thymopoiesis to proceed normally. After T lineage commitment, Spi-B function is necessary for efficient development of fetal DN thymocytes to the DP stage, but if Spi-B is too highly expressed or is not down-regulated normally, development is perturbed, with the outcome depending on the expression level.

Our analysis represents the first demonstration that deviation from the prescribed pattern of Spi-B expression results in a dose-dependent perturbation of development in the thymus; and that the dose-dependence is also observed in purified DN3 thymocytes. These findings extend those obtained previously following transduction of mixed precursor populations (44, 59). Retroviral transduction of Spi-B into PU.1-deficient fetal liver precursors resulted in a dose-dependent rescue of development, with lower levels of Spi-B rescuing B lymphoid development and higher levels promoting precursors to adopt a myeloid fate (59). Moreover, retroviral transduction of Spi-B into human thymocytes resulted in a modest promotion of DC development after protracted incubation periods (44). Although these authors also noted a block in T lineage development, they suggested that Spi-B overexpression had no effect on cells after T lineage commitment has occurred. This interpretation is not supported by our analysis of murine T cell precursors, because transduction of T lineage-committed DN3 cells caused either diversion to the DC lineage or arrest at the β-selection checkpoint, depending on the Spi-B expression level.

Although the enforced expression of Spi-B had previously been reported to perturb development, there was little understanding of the mechanistic basis for this effect (44, 60). We now demonstrate that the blockade of β-selection by enforced expression of Spi-B does not appear to result from interference with pre-TCR expression; rather, it appears to result from effects either on pre-TCR signaling or on execution of the genetic program underlying β-selection. Our initial inclination that Spi-B might be affecting pre-TCR expression arose as a result of overlap in the gene targets modulated by Spi-B and PU.1 (61–63). PU.1 overexpression had previously been reported to suppress RAG and pre-Tα mRNA levels (43, 54). Consequently, if Spi-B were to suppress these genes, Spi-B overexpression might interfere with *TCRβ* rearrangement or pre-TCR assembly; however, we found no effect of Spi-B overexpression on the frequency of *TCRβ*-expressing DN3 thymocytes (Fig. 7) or on expression of pre-TCR complexes by a thymic lymphoma (data not shown). Also by analogy with PU.1, we examined the effect of Spi-B on expression of several genes important for early thymocyte development, *GATA-3*, c-*myb*, and *hes-1*, because these genes are suppressed by PU.1 overexpression (54, 64–66). Spi-B overexpression did not affect the expression of these genes (data not shown); however, posttranslational effects have not been excluded. PU.1 has been reported to interfere with GATA-1 binding to DNA and because of the high degree of homology might also interfere with GATA-3 function (67, 68). Nevertheless, the N-terminal domain of PU.1, which is involved in GATA-1 interaction is not conserved in Spi-B, decreasing the likelihood that GATA-3 is a Spi-B target in this context (67, 68). Instead, our analysis revealed that Spi-B affected the induction of Egr transcription factors. Enforced expression of Spi-B interfered with induction of Egr2 and Egr3 with the most significant effect being on Egr3; however, Spi-B was not found to affect Egr1 induction. The impairment of Egr induction appears to be an important aspect of the Spi-B-mediated blockade of differentiation, because the blockade can be effectively overcome by enforced expression of Egr2 and -3. Although it is not clear whether Spi-B overexpression is having a direct effect on the promoters of Egr2 and -3, there is a consensus, composite Spi-B/IFN regulatory factor (IRF)-4/8 binding site in both the mouse and human Egr3 promoters (data not shown) (69). The mechanistic basis for the effect of Spi-B on Egr2 and -3 induction is currently under investigation.

The ability of enforced expression of Spi-B to block development beyond the β-selection checkpoint underscores the importance of Spi-B down-modulation during this developmental transition; however, the importance of Spi-B induction at the DN3 stage is less clear. Spi-B deficiency does not appear to impair development beyond the β-selection checkpoint to the DP stage in adult mice, but does cause a delay in development of fetal thymocytes. The relatively modest effect of Spi-B deficiency on development beyond the β-selection checkpoint may result from functional redundancy with PU.1, because Spi-B and PU.1 have been shown to exhibit functional redundancy in some developmental contexts (50, 51). Although the mechanistic basis for the observed

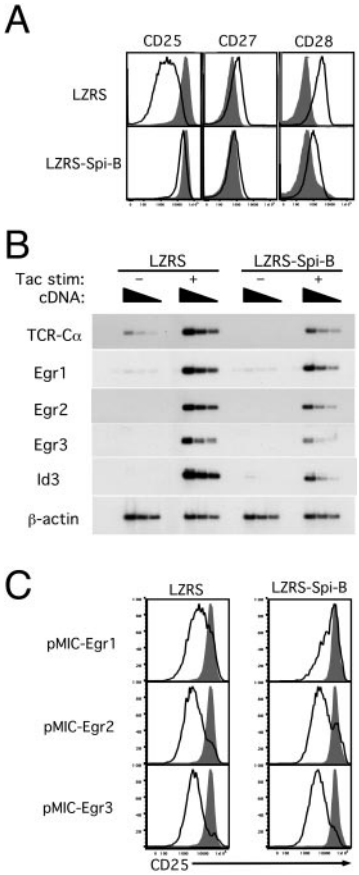

FIGURE 8. Enforced expression of Spi-B blocks Scid.adh differentiation by antagonizing the induction of Egr proteins. *A–C*, Scid.adh TAC:CD3ϵ cells were transduced with either LZRS or LZRS-Spi-B. After 4–5 days, the GFP^+ cells were isolated by flow cytometry. Note: To eliminate those cells that had been diverted to the DC lineage, only those LZRS-Spi-B-transduced cells that were $CD11c^-$ were included in this analysis. *A*, LZRS- and LZRS-Spi-B-transduced cells were cultured for 24 h in the presence (black line) or absence (filled gray) of plate-bound anti-TAC Ab, and then their differentiation was assessed by flow cytometry using the indicated Ab. *B*, Changes in expression of the indicated genes was assessed in cells treated as in *A* by RT-PCR Southern blotting as above. *C*, LZRS- and LZRS-Spi-B-transduced Scid.adh TAC:CD3ϵ were superinfected with empty pMIC retrovirus (filled gray) or pMIC virus encoding the indicated Egr proteins (black line). After 30 h, the effect on CD25 expression was analyzed by FACS on electronically gated CFP^+ population.

delay remains to be established, we advance the following speculation. It is possible that Spi-B serves a prosurvival role in DN3 thymocytes before the onset of pre-TCR signals, which then down-regulate Spi-B when DN3 thymocytes transition from cytokine dependence to dependence upon pre-TCR signaling. Accordingly, Spi-B induction may modulate expression of the IL-7R following PU.1 down-modulation during the DN2–DN3 transition (43, 54). Although we found no change in IL-7R expression in Spi-B-deficient DN3 thymocytes, the low level of IL-7R expression in normal DN3 make any alterations in expression difficult to detect (59). The mechanistic basis for delayed development in Spi-B-deficient, and Spi-B/PU.1 double-deficient thymocytes will be the focus of future studies.

Our finding that marked overexpression (~30 times endogenous) of Spi-B is able to redirect a thymic lymphoma and even purified DN3 thymocytes to the DC lineage suggests that Spi-B is capable of overcoming those molecular alterations that underlie commitment to the T cell lineage. The mechanistic basis whereby Spi-B diverts thymocytes to the DC lineage remains unclear, but is likely to involve cooperative interactions with the Spi-B/PU.1 cofactor, IRF-8, because IRF-8 deficiency selectively blocks development of $CD8^+$ thymic DC (70, 71). To our knowledge, this is the first example of any treatment that is capable of diverting DN3 thymocytes away from the T lineage (4, 5). Furthermore, Spi-B (but not PU.1) is highly expressed in human plasmacytoid DC, the closest human counterpart to murine $CD8^+$ thymic DC (44); and short hairpin RNA-mediated knockdown of Spi-B in human fetal precursors has recently been reported to impair development of plasmacytoid DC (72). Nevertheless, the role of Spi-B in development of thymic DC in the mouse remains unclear, because Spi-B deficiency did not grossly perturb DC development in the thymus. Previously published analysis of the PU.1-deficient mouse line used in our study suggested that development of thymic DC was delayed, but that DC were present after day 16.5 (52). In contrast, analysis of an independently generated PU.1-deficient mouse line suggested that PU.1 deficiency blocked development of both myeloid and lymphoid DC (53). To address this controversy and the possibility that Spi-B and PU.1 might both support DC development in a functionally redundant manner, we analyzed both PU.1-deficient and PU.1-Spi-B double-deficient mice. Our analysis revealed that DC were absent from day 18.5 fetal thymi from both the PU.1-deficient mice described above and from PU.1-Spi-B double-deficient mice. It is unclear why previous analysis of the PU.1-deficient mice revealed the presence of thymic DC, but may relate to differences in mouse strain, because the previous analysis was performed on a mixed 129 × B6 background, whereas the mice used in our experiments have been backcrossed to C57BL/6 for 10 generations. This possibility is currently being addressed. Therefore, despite the fact that short hairpin RNA-mediated knockdown of Spi-B impairs development of human lymphoid DC (72) and enforced expression of Spi-B can induce mouse DN3 to adopt a DC fate, our data suggest that Spi-B cannot compensate for the loss of PU.1 in supporting DC development in the mouse. We presume that is because Spi-B expression is restricted to the DN3 fraction that no longer displays DC progenitor activity and because the level of Spi-B expressed by DN3 is below that required to divert those cells to the DC lineage. Although these data do not provide compelling support for a role for Spi-B in development of murine thymic DC, they also do not rule out the possibility that Spi-B might act to modulate DC development/maturation beyond the stage at which development is arrested by PU.1 deficiency. The production of Spi-B-deficient and conditional PU.1-null mice will be required to address this possibility.

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Disclosures

The authors have no financial conflict of interest.

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