The EMBO Journal Vol. 21 No. 9 pp. 2220–2230, 2002

Spi-B can functionally replace PU.1 in myeloid but not lymphoid development

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Mature macrophages, neutrophils and lymphoid cells do not develop in *PU.1*$^{-/-}$ mice. In contrast, mice lacking the highly related protein Spi-B generate all hematopoietic lineages but display a B-cell receptor signaling defect. These distinct phenotypes could result from functional differences between PU.1 and Spi-B or their unique temporal and tissue-specific expression (PU.1: myeloid and B cells; Spi-B: B cells only). To address this question, we introduced the Spi-B cDNA into the murine *PU.1* locus by homologous recombination. In the absence of PU.1, Spi-B rescued macrophage and granulocyte development when assayed by *in vitro* differentiation of embryonic stem cells. Adherent, CD11b^{+}/F4/80^{+} cells capable of phagocytosis were detected in *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ embryoid bodies, and myeloid colonies were present in hematopoietic progenitor assays. Despite its ability to rescue myeloid differentiation, Spi-B did not rescue lymphoid development in a *RAG-2*$^{-/-}$ complementation assay. These results demonstrate an important difference between PU.1 and Spi-B. Careful comparison of these Ets factors will delineate important functional domains of PU.1 involved in lymphocyte lineage commitment and/or maturation.

Keywords: hematopoiesis/PU.1/Spi-B

Introduction

The Ets family of DNA-binding proteins is a highly conserved group of transcriptional regulators with important developmental functions in all metazoans examined (Graves and Petersen, 1998). All family members share sequence conservation in their DNA-binding domains (the Ets domain). The Spi transcription factors, PU.1 (Spi-1), Spi-B, Spi-C and Spi-D, represent a divergent subfamily of Ets transcription factors only identified in vertebrates thus far. Their expression appears to be restricted to the hematopoietic lineages. PU.1 is expressed predominantly in granulocytes, macrophages and B cells, with low levels detected in early T cells and erythrocytes (Klemsz *et al*., 1990; Galson *et al*., 1993; Hromas *et al*., 1993; Chen *et al*., 1995). Spi-B is detected in early thymocytes and B cells (Su *et al*., 1996; Anderson *et al*., 1999). Spi-C is expressed predominantly in B cells, with some expression in myeloid cells (Bemark *et al*., 1999; Hashimoto *et al*., 1999). Lastly, Spi-D has been identified recently in *Xenopus laevis* and *Raja eglanteria* (skate), but tissue-specific expression has not yet been characterized (Shintani *et al*., 2000; Anderson *et al*., 2001). Within the subfamily, PU.1, Spi-B and Spi-D are more related to each other than to Spi-C. Along with sequence conservation in the Ets DNA-binding domain, PU.1, Spi-B and Spi-D share a conserved PEST domain which contains a critical phosphorylation site required for interaction with IRF family members PIP and ICSBP (Eisenbeis *et al*., 1995; Brass *et al*., 1996). PU.1 differs from Spi-B and Spi-D at the N-terminus, exhibiting a divergent transactivation domain (Rao *et al*., 1999b) that includes a glutamine-rich region essential for the generation of myeloid cells (Fisher *et al*., 1998).

We have engineered targeted deletions of *PU.1* and *Spi-B* genes in mice that yield surprisingly different phenotypes. Two independent lines of *PU.1*$^{-/-}$ mice have been generated and each exhibits severe hematopoietic defects (Scott *et al*., 1994; McKercher *et al*., 1996). Neither mutant develops fetal macrophages, granulocytes or B lymphocytes. *PU.1*$^{-/-}$ mice generated by Scott *et al*. die at day 18.5 of gestation precluding an examination of postnatal hematopoiesis. In contrast, the *PU.1*$^{-/-}$ mice generated by McKercher *et al*. survive to birth and live up to 3 weeks if maintained on antibiotics. T lymphocytes and immature, non-functional granulocytic cells are detected in such mice. However, there was no evidence of definitive macrophage and B-lymphocyte development.

Deletion of Spi-B results in a milder defect (Su *et al*., 1997). *Spi-B*$^{-/-}$ mice are viable and although all hematopoietic lineages are present, B lymphocytes are functionally compromised. Cross-linking of the B-cell antigen receptor (BCR) results in reduced B-lymphocyte proliferation, increased apoptosis and reduced phosphorylation of downstream substrates (Garrett-Sinha *et al*., 1999). When challenged with a T-dependent antigen, antibody production is diminished and splenic germinal centers develop poorly. Since Spi-B and PU.1 are co-expressed in B lymphocytes, these two mutant strains were crossed to look for potential redundancy. The *PU.1* mutation used in this study is lethal, so *PU.1*$^{+/-}$*Spi-B*$^{-/-}$ mice were examined. Compound mutant mice are more defective in BCR-mediated signaling and germinal center formation than the *Spi-B*$^{-/-}$ animals. These results demonstrate functional overlap and/or a genetic interaction between PU.1 and Spi-B (Garrett-Sinha *et al*., 1999). Similar crosses with non-Spi subfamily members, *Ets-1* and *Elf-1*, did not reveal any redundancy or genetic interactions with *PU.1* or *Spi-B* (Garrett-Sinha *et al*., 2001).

To address directly the question of functional redundancy between Ets family members, we introduced Spi-B

or Ets-1 cDNAs into the *PU.1* locus via gene targeting. This approach allows a stringent determination of whether either factor can functionally replace PU.1. We report here that Spi-B, but not Ets-1, can rescue myelopoiesis (granulocyte and macrophage development). Interestingly, Spi-B could not replace PU.1 during lymphopoiesis. The inability of Spi-B to rescue lymphocyte development supports the conclusion that PU.1 has unique functional abilities that cannot be performed by Spi-B.

Results

Generation of homozygous knock-in clones

To investigate further the redundancy of PU.1 and Spi-B, we directly tested the ability of Spi-B to carry out the activities of PU.1 in hematopoiesis. The domain structure of these two factors is very similar (see Figure 1), sharing conserved Ets DNA-binding and PEST domains. The PEST region is involved in protein–protein interactions. We also tested the ability of the highly divergent Ets-1 protein to substitute for PU.1, in case hematopoietic development requires an Ets family member and not specifically a Spi factor. Embryonic stem (ES) cell clones expressing Spi-B or Ets-1 in place of PU.1 were generated using a targeting vector that deletes ~1 kb of genomic sequence from the *Sac*I site of exon 1 to a downstream intronic *Bam*HI site (Figure 2A). The non-coding sequence of exon 1 is retained up to the initiating methionine. Murine PU.1, human Spi-B or human Ets-1 cDNA was inserted into the *Sac*I site 6 bp upstream of the endogenous PU.1 initiating methionine. These constructs along with a 'knock-out' construct (no cDNA included) were introduced into RW4 ES cells. Either PU.1, Spi-B or Ets-1 cDNA and/or a PGK-Neo expression cassette replace the deleted genomic sequence. LoxP sites surround the PGK::Neo cassette to allow for Cre-mediated excision. Heterozygous cells were detected by Southern blot assay and were recloned in increasing concentrations of G418 to select for homozygous mutant clones.

To determine if the inserted cDNAs were being expressed, heterozygous ES cell clones were differentiated *in vitro* under conditions that promote hematopoietic development. After 11 days of culture, embryoid body (EB) RNA was assayed for cDNA expression by RT–PCR.

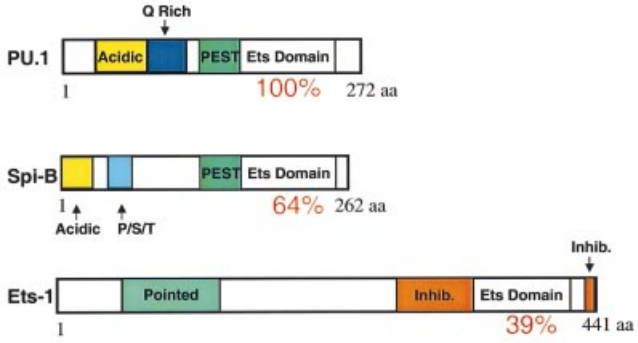

Fig. 1. Comparison of functional domains between PU.1, Spi-B and Ets-1.

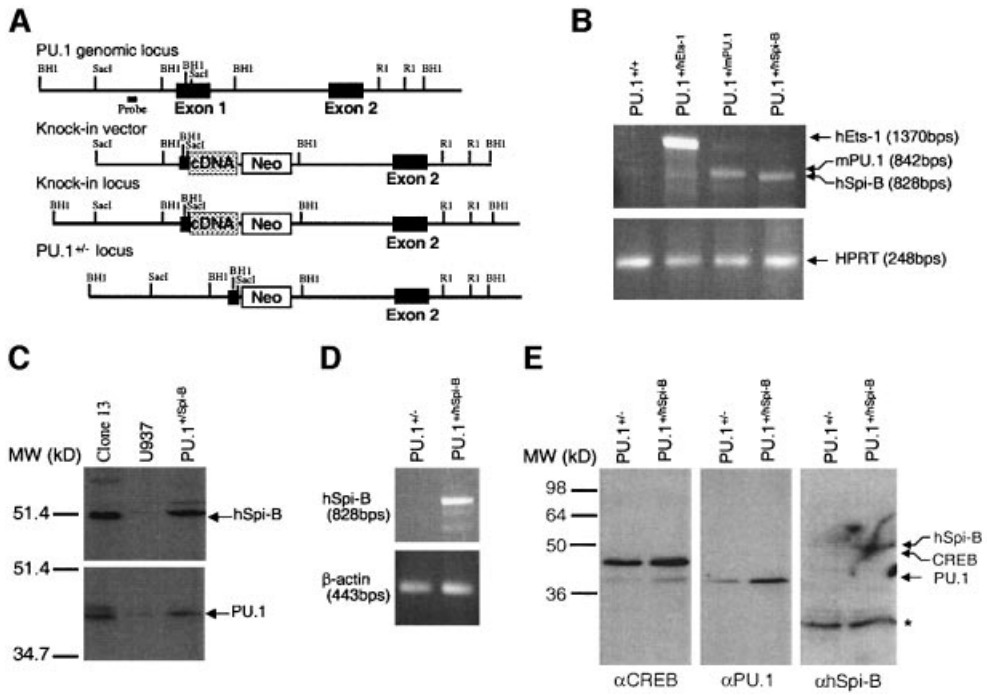

Fig. 2. Generation of homozygous knock-in ES cell clones. (**A**) Schematic representation of the targeting strategy. (**B**) RT–PCR of RNA isolated from *in vitro* differentiated heterozygous knock-in clones. Primers correspond to the 5′ prime epitope tag and the 3′ prime polyadenylation sequences. Forty cycles of PCR were used to amplify target cDNAs. (**C**) Western blot of protein isolated from clone 13, U937 and splenic *PU.1*$^{+/Spi\text{-}B}$ cells. A total of 500 000 cell equivalents were loaded per lane. The blot was first probed with anti-human Spi-B antibody (Su *et al*., 1996) and subsequently reprobed with anti-PU.1 antibody (Santa Cruz Biotechnology). (**D**) RT–PCR of RNA isolated from splenic B cells. The B-cell preparation was >95% CD19^{+}/B200^{+} as determined by FACS analysis. Amplification of cDNA was performed as previously stated. (**E**) Western blot of lysates prepared from splenic B cells. Approximately 150 μg of total cellular protein was loaded per well. The human Spi-B (h-Spi-B) antibody does not recognize murine Spi-B. The blot was first probed with anti-hSpiB and subsequently reprobed with anti-PU.1 and anti-CREB. CREB expression is shown to demonstrate approximately equivalent loading of cell lysate. The asterisk denotes a non-specific immunoreactive band.

Appropriate size PCR fragments were detected in EB RNA prepared from the three knock-in clones, but no product was amplified in wild-type EBs (Figure 2B) or control PCRs of RNA samples not reverse transcribed (data not shown). To confirm further that the targeting strategy results in expression of the inserted cDNA, $PU.1^{+/Spi\text{-}B}$ mice generated from targeted ES cells were examined for Spi-B expression by western blot. A Spi-B immunoreactive band detected in $PU.1^{+/Spi\text{-}B}$ splenic cell extract co-migrated with human Spi-B (hSpi-B) in clone 13 human B cells (Figure 2C). As expected, no Spi-B immunoreactivity was detected in U937 myeloid cells. Importantly, this antibody is specific for hSpi-B (Su *et al*., 1996) and exclusively binds Spi-B expressed from the murine *PU.1* locus. Since both the EBs and the spleen predominantly contain myeloid cells, we also isolated splenic B cells from $PU.1^{+/Spi\text{-}B}$ mice to assay Spi-B expression. The isolated cells were ~95% $CD19^+/B220^+$ (data not shown). RT–PCR using the same primers from Figure 2B detected expression of the knocked-in hSpi-B allele (Figure 2D), and hSpi-B protein was detected from $PU.1^{+/Spi\text{-}B}$, but not $PU.1^{+/-}$ B cells (Figure 2E). These results (Figure 2B–E) demonstrate that our targeting strategy results in expression of the inserted cDNA. Lastly, no PU.1 protein was detected in EBs derived from $PU.1^{-/-}$, $PU.1^{Ets\text{-}1/Ets\text{-}1}$ or $PU.1^{Spi\text{-}B/Spi\text{-}B}$ ES cells (data not shown). In all assays, the new knock-out allele behaves identically to our previous null allele (Scott *et al*., 1994; Olson *et al*., 1995). Therefore, phenotypes resulting from the knocked-in alleles are due to each expressed cDNA and not to residual PU.1 protein expression.

Spi-B, but not Ets-1, can replace PU.1 function to generate macrophages in vitro

$PU.1^{+/+}$, $PU.1^{-/-}$, $PU.1^{PU.1/PU.1}$, $PU.1^{Ets\text{-}1/Ets\text{-}1}$ and $PU.1^{Spi\text{-}B/Spi\text{-}B}$ ES cells were differentiated *in vitro* in methylcellulose containing hematopoietic cytokines [stem cell factor (SCF), interleukin (IL)-1, IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF) and erythropoietin]. After 11–16 days of incubation, cultures were assayed for the presence of macrophages. EBs were evaluated by cell morphology and immunohistochemical detection of the myeloid markers F4/80 and CD11b. Figure 3A, D, G, J and M depicts cytospin preparations of *in vitro* differentiated EBs. Only $PU.1^{PU.1/PU.1}$ and $PU.1^{Spi\text{-}B/Spi\text{-}B}$ EBs produced cells morphologically similar to macrophages derived from $PU.1^{+/+}$ EBs.

To confirm that macrophages were produced, cells derived from 11- to 16-day EBs were plated overnight and adherent cells stained with antibodies to the myeloid-specific markers F4/80 and CD11b. Consistent with the morphological evaluation, only the PU.1 knock-in (Figure 3H) and Spi-B knock-in (Figure 3N) cultures contained adherent cells positive for F4/80, denoted by

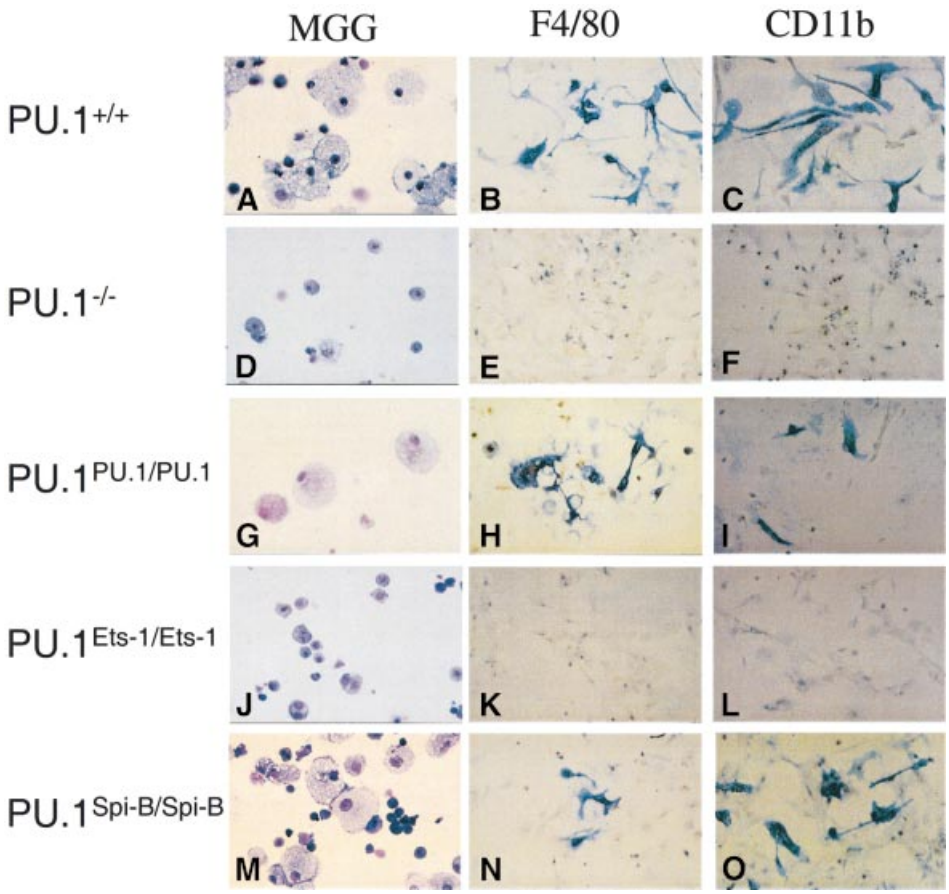

Fig. 3. Identification of macrophages by histological and immunocytochemical staining. (**A**, **D**, **G**, **J** and **M**) May–Grunwald–Giemsa staining of cytocentrifuged day 11–16 EBs. (**B**, **E**, **H**, **K** and **N**) Adherent cells harvested from day 11–16 trypsinized EBs and stained with antibody to F4/80. (**C**, **F**, **I**, **L** and **O**) Adherent cells stained with antibody to CD11b. Blue staining indicates the presence of antigen. Magnification = 400×.

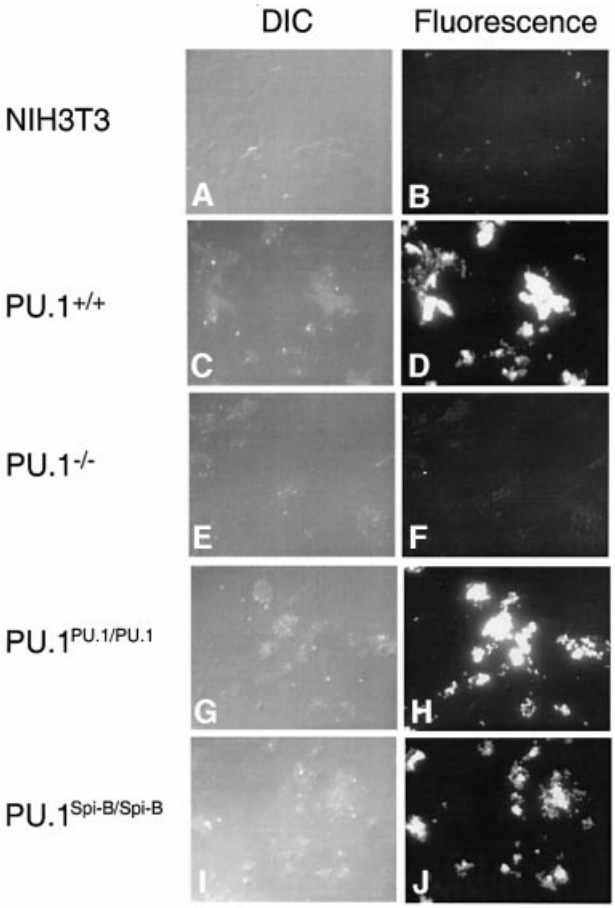

Fig. 4. Phagocytosis of opsonized zymosan. Adherent cells harvested from day 11–16 trypsinized EBs were incubated with opsonized fluorescein-labeled zymosan particles for 30 min at 37°C. Cells were washed in PBS and fixed in 3.7% formaldehyde. Photographs show differential interference contrast (DIC) and corresponding fluorescent images.

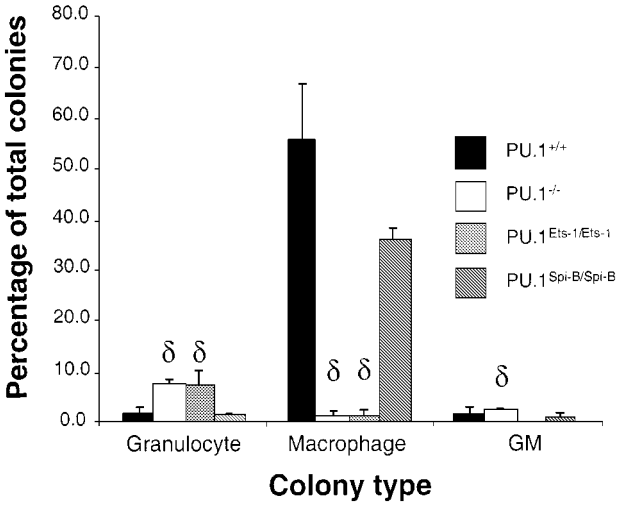

Fig. 5. Hematopoietic colony formation. Single-cell suspensions from day 9–11 EBs were plated into a hematopoietic cytokine-rich methylcellulose medium (Methocult 3434, Stem Cell Technologies) and allowed to differentiate. Hematopoietic colonies were scored after 5–7 days of differentiation. A 'δ' denotes that these colonies after further examination are not mature myeloid colonies. Each genotype was assayed in triplicate and the error bars represent standard deviation from the mean percentage of colonies.

cell-associated blue staining. These same cultures were also CD11b$^+$ (Figure 3I and O). No F4/80 or CD11b staining was detected in cultures derived from the *PU.1*$^{-/-}$ or *PU.1*$^{Ets\text{-}1/Ets\text{-}1}$ ES cells (Figure 3E, F, K and L). Furthermore, these results were consistent amongst multiple independent *PU.1*$^{-/-}$, *PU.1*$^{PU.1P/PU.1}$, *PU.1*$^{Ets\text{-}1/Ets\text{-}1}$ and *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ clones analyzed. In addition, removal of the *neo*^{R} cassette by Cre-mediated excision did not change the observed phenotypes (data not shown).

Along with immunohistochemical analysis, EB-derived adherent cells were examined for their ability to phagocytose fluorescent zymosan particles. NIH 3T3 cells were plated as a negative control. Cells were incubated with opsonized fluorescein isothiocyanate (FITC)–zymosan particles for 30 min at 37°C. After incubation, cells were washed several times to remove non-phagocytosed particles. No cell-associated fluorescence was observed with NIH 3T3 cells (Figure 4A and B) and very little fluorescence was associated with *PU.1*$^{-/-}$ cells (Figure 4E and F). Fluorescent particles were highly associated with cells from *PU.1*$^{+/+}$, *PU.1*$^{PU.1/PU.1}$ and *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ cultures, indicating that these cells were capable of phagocytosis (Figure 4C, D, G, H, I and J). From the EB analysis, we conclude that Spi-B can replace PU.1 in generating macrophages and that these cells are functionally competent for both adherence and phagocytosis.

PU.1$^{Spi\text{-}B/Spi\text{-}B}$ *ES cells partially rescue granulocytic development in vitro*

Primary differentiation of ES cells into EBs demonstrated that Spi-B, but not Ets-1, rescued macrophage development but did not address whether Ets-1 or Spi-B generated granulocytic cells. To assess granulocytic differentiation, primary EB cultures were replated into hematopoietic cytokine-rich media (SCF, IL-3, IL-6 and erythropoietin) and cultured for an additional 5–7 days in standard colony-forming assays that allow quantitative assessment of hematopoietic progenitors. Along with analysis of granulocytic development, the increased sensitivity of progenitor assays also potentially could reveal the ability of Ets-1 to generate macrophages.

Unexpectedly, myeloid (macrophage and granulocyte) colonies were detected in all cultures (Figure 5). However, the number of CFU-M and the cells per colony in *PU.1*$^{-/-}$ and *PU.1*$^{Ets\text{-}1/Ets\text{-}1}$ cultures were greatly reduced compared with *PU.1*$^{+/+}$ and *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ cultures. Furthermore, only wild-type and Spi-B knock-in colonies contained mature macrophages when examined by cytocentrifugation and histochemical staining. A representative cell from each genotype is shown in Figure 6A. Although the cells from *PU.1*$^{-/-}$ and *PU.1*$^{Ets\text{-}1/Ets\text{-}1}$ cultures may be of myeloid origin, they clearly do not resemble mature macrophages and do not express macrophage-specific genes. RT–PCR analysis performed on RNA derived from the clonogenic cultures revealed that all genotypes expressed CD18, consistent with previous results with *PU.1*$^{-/-}$ ES cells (Olson *et al.*, 1995). However, only *PU.1*$^{+/+}$ and *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ clones expressed high levels of CD11b and macrophage colony-stimulating factor (M-CSF) receptor transcripts (Figure 6B).

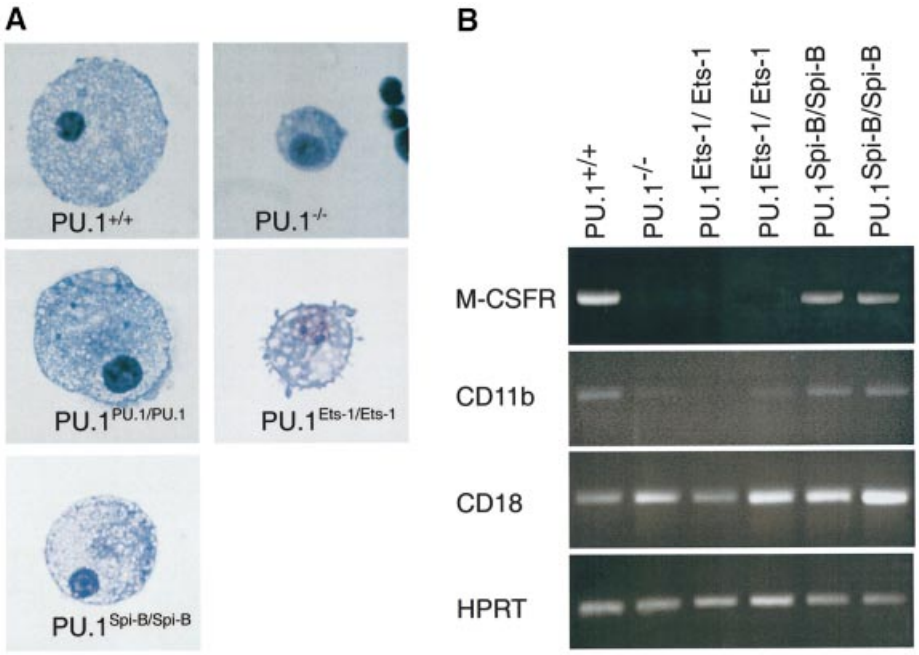

Fig. 6. Examination of macrophage colonies. (**A**) Macrophage colonies from each genotype were isolated from methylcellulose cultures, cytocentrifuged onto glass slides and stained with May–Grunwald–Giemsa. Magnification = 1000×. (**B**) RNA was isolated from hematopoietic cultures and subjected to RT–PCR analysis. Cultures derived from two independent clones of $PU.1^{Ets\text{-}1/Ets\text{-}1}$ and $PU.1^{Spi\text{-}B/Spi\text{-}B}$ ES cells were used. Primer pairs specific for M-CSFR, CD11b or CD18 did not generate appropriate size fragments when genomic DNA was used as the template (data not shown).

Although the morphology of $PU.1^{-/-}$ and $PU.1^{Ets\text{-}1/Ets\text{-}1}$ granulocyte colonies (CFU-G) appeared grossly normal, histological staining of cytospin preparations revealed differences between colonies derived from these genotypes and $PU.1^{+/+}$ CFU-G. Furthermore, $PU.1^{Ets\text{-}1/Ets\text{-}1}$ granulocytic cells were identical to those from $PU.1^{-/-}$ cultures (Figure 7A). Phenotypically, $PU.1^{Ets\text{-}1/Ets\text{-}1}$ and $PU.1^{-/-}$ CFU-G appeared to be immature granulocytes: the cytoplasm retained a dark purple color indicating a basic pH, and the majority of nuclei were not segmented. This result differs from our previous studies of $PU.1^{-/-}$ yolk sac and fetal liver hematopoiesis in which no myeloid colonies were detected (Scott *et al*., 1994; Olson *et al*., 1995). However, the morphology appears to be similar to that of neutrophils isolated from $PU.1^{-/-}$ animals generated by McKercher *et al*. (Anderson *et al*., 1998) and to granulocytic cells produced by a hematopoietic cell line derived from our $PU.1^{-/-}$ mice (DeKoter *et al*., 1998). In contrast, $PU.1^{Spi\text{-}B/Spi\text{-}B}$ granulocytes more closely resembled those isolated from wild-type and *PU.1* knock-in cultures: the nuclei were clearly segmented and the cytoplasm was less basic (Figure 7A). The morphologies of $PU.1^{+/+}$ and $PU.1^{Spi\text{-}B/Spi\text{-}B}$ cells were not identical, but clearly Spi-B had an effect on the differentiation of granulocytic cells.

In addition to cellular morphology, we examined the hematopoietic colony assays for expression of granulocyte-specific genes using RT–PCR. RNA was prepared from an entire 30 mm plate containing colonies of several hematopoietic lineages. Granulocytic cells from $PU.1^{-/-}$ mice were shown previously to express myeloperoxidase (MPO), but not lysozyme and secondary granule genes such as lactoferrin (Anderson *et al*., 1998). We assayed our cultures for the expression of MPO, lysozyme, lactoferrin, gelatinase B and CCAAT/enhancer-binding protein ε (C/EBPε). C/EBPε is expressed late in granulocytic differentiation, and the phenotype of $C/EBP\varepsilon^{-/-}$ neutrophils is similar to that of $PU.1^{-/-}$ (Morosetti *et al*., 1997; Yamanaka *et al*., 1997a,b); however, its expression had not been assayed previously in PU.1 null cells. As expected, MPO expression was detected in cultures of all genotypes (Figure 7C). Some variability in expression was seen which may be due to different numbers of granulocytes present in each culture and not necessarily due to decreased expression in individual granulocytic cells. We also examined MPO protein activity using an enzymatic assay. MPO activity mirrored gene expression data as $PU.1^{-/-}$ cells were marginally positive but much more MPO activity was detected in the $PU.1^{+/+}$ cells, as indicated by the increase in dark brown staining (Figure 7B). An intermediate level of MPO activity was detected in $PU.1^{Spi\text{-}B/Spi\text{-}B}$ cells. To our surprise, C/EBPε transcripts could be detected in cells of all genotypes, including $PU.1^{-/-}$ (Figure 7C). In contrast, it was difficult to amplify gelatinase B even in $PU.1^{+/-}$ cultures (data not shown). Therefore, C/EBPε and gelatinase B expression could not be used as an indicator of rescue. Conversely, lysozyme expression was seen consistently in $PU.1^{+/-}$ cultures (Figure 7C) but not $PU.1^{-/-}$ cultures as reported by Anderson *et al*. (1998). Importantly, lysozyme was detected in both $PU.1^{Spi\text{-}B/Spi\text{-}B}$ clones examined, but not in either $PU.1^{Ets\text{-}1/Ets\text{-}1}$ clone (Figure 7C). This result is striking since more CFU-G colonies are produced by $PU.1^{-/-}$ and $PU.1^{Ets\text{-}1/Ets\text{-}1}$ cultures (see Figure 5). Lastly, we examined the expression of lactoferrin. Lactoferrin mRNA was clearly detected in $PU.1^{+/-}$ cultures, but only very faint PCR products were amplified in RNA prepared

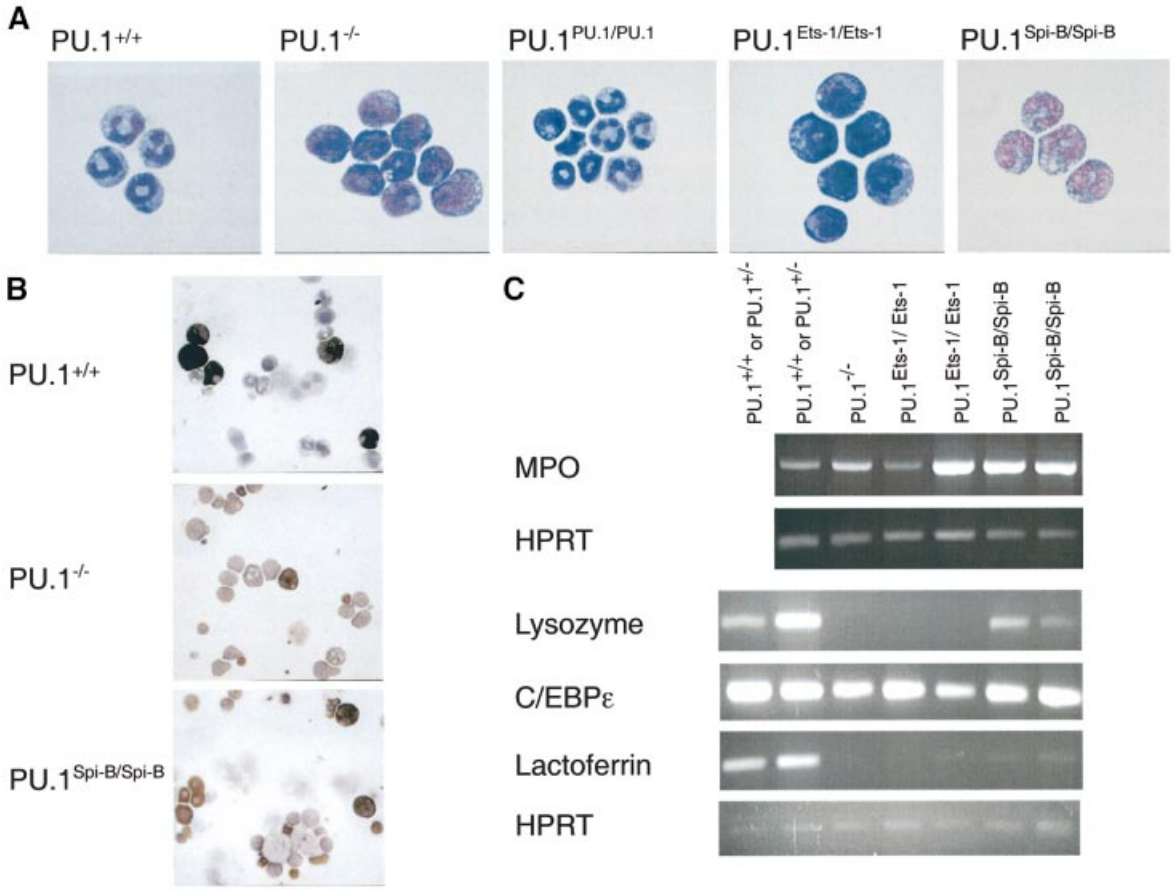

Fig. 7. Examination of granulocyte colonies. (**A**) Granulocyte colonies from each genotype were isolated from methylcellulose cultures, cytocentrifuged onto glass slides and stained with May–Grunwald–Giemsa. Magnification = 1000×. (**B**) Cytospin preparations were stained for MPO activity using a diagnostic kit (Sigma). Dark brown staining indicates the presence of MPO activity. Magnification = 200×. (**C**) RNA isolated from entire hematopoietic cultures was subjected to RT–PCR analysis. Cultures derived from two independent clones of *PU.1*$^{+/-}$, *PU.1*$^{Et\text{-}1/Ets\text{-}1}$ and *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ ES cells were used. For the upper two panels (MPO and HPRT), RNA prepared from one *PU.1*$^{+/+}$ culture was used instead of two *PU.1*$^{+/-}$ cultures. Primer pairs specific for MPO, lysozyme, lactoferrin, C/EBPε and HPRT did not generate the predicted fragment when genomic DNA and/or mock reverse-transcribed RNA was used as the template (data not shown).

from the other genotypes (Figure 7C). Therefore, based on morphology, MPO activity and lysozyme expression data, we conclude that Spi-B partially rescues the *PU.1*$^{-/-}$ defect in granulocytic development. However, the lack of lactoferrin expression in *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ neutrophils suggests that this rescue of phenotype is incomplete.

Spi-B cannot replace PU.1 in the generation of lymphocytes

PU.1 is essential for the development of both the myeloid and lymphoid lineages (Scott *et al.*, 1994; McKercher *et al.*, 1996). Since Spi-B could substitute for PU.1 in the generation of myeloid lineages, we wanted to test whether Spi-B could also replace PU.1 in the production of lymphocytes. We assessed the ability of *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ cells to differentiate into lymphoid cells using the *RAG-2*$^{-/-}$ blastocyst complementation assay (Chen *et al.*, 1993). *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$, *PU.1*$^{-/-}$ and *PU.1*$^{+/-}$ ES cells were injected into blastocysts obtained from *RAG-2*$^{-/-}$ mice. The resultant chimeras were then assayed for the presence of B and T lymphocytes. The *RAG-2* mutation blocks lymphoid development at the pro-B- and pro-T-cell stage; therefore, any mature lymphocytes detected in chimeric mice must be of donor ES cell origin.

PU.1$^{+/-}$ but not *PU.1*$^{-/-}$ cells were able to generate IgM$^+$/B220$^+$ cells in the spleen. As shown previously by flow cytometry in C57BL/6 chimeric mice (Scott *et al.*, 1997), *PU.1*$^{-/-}$ cells were unable to contribute to either T- or B-cell populations in *RAG-2*$^{-/-}$ chimeras. However, *PU.1*$^{+/-}$ ES cells rescued both B- and T-cell development in *RAG-2*$^{-/-}$ mice (Figure 8). B220$^+$/IgM$^+$ cells were detected in the bone marrow of *PU.1*$^{+/-}$ chimeras. Furthermore, B cells exited to the periphery and engrafted the spleen as both B220$^+$/IgM$^+$ and IgM$^+$/IgD$^+$ cells. T cells engrafted the thymus as CD4$^+$/CD8$^+$ double-positive (DP) cells and CD4$^+$ or CD8$^+$ single-positive (SP) cells. Furthermore, SP T cells were detected in the spleen of chimeric mice.

In direct contrast, no B220$^+$/IgM$^+$, CD4$^+$ or CD8$^+$ cells were detected in the various hematopoietic organs of any *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ chimera ($n = 17$; Figure 8). Two independent ES cell clones, one with the *neo* cassette removed, were tested, and chimerism ranged from 5 to 60% as judged by coat color (Table I). Surprisingly, significant lethality was observed in chimeric mice, with ES cell contributions

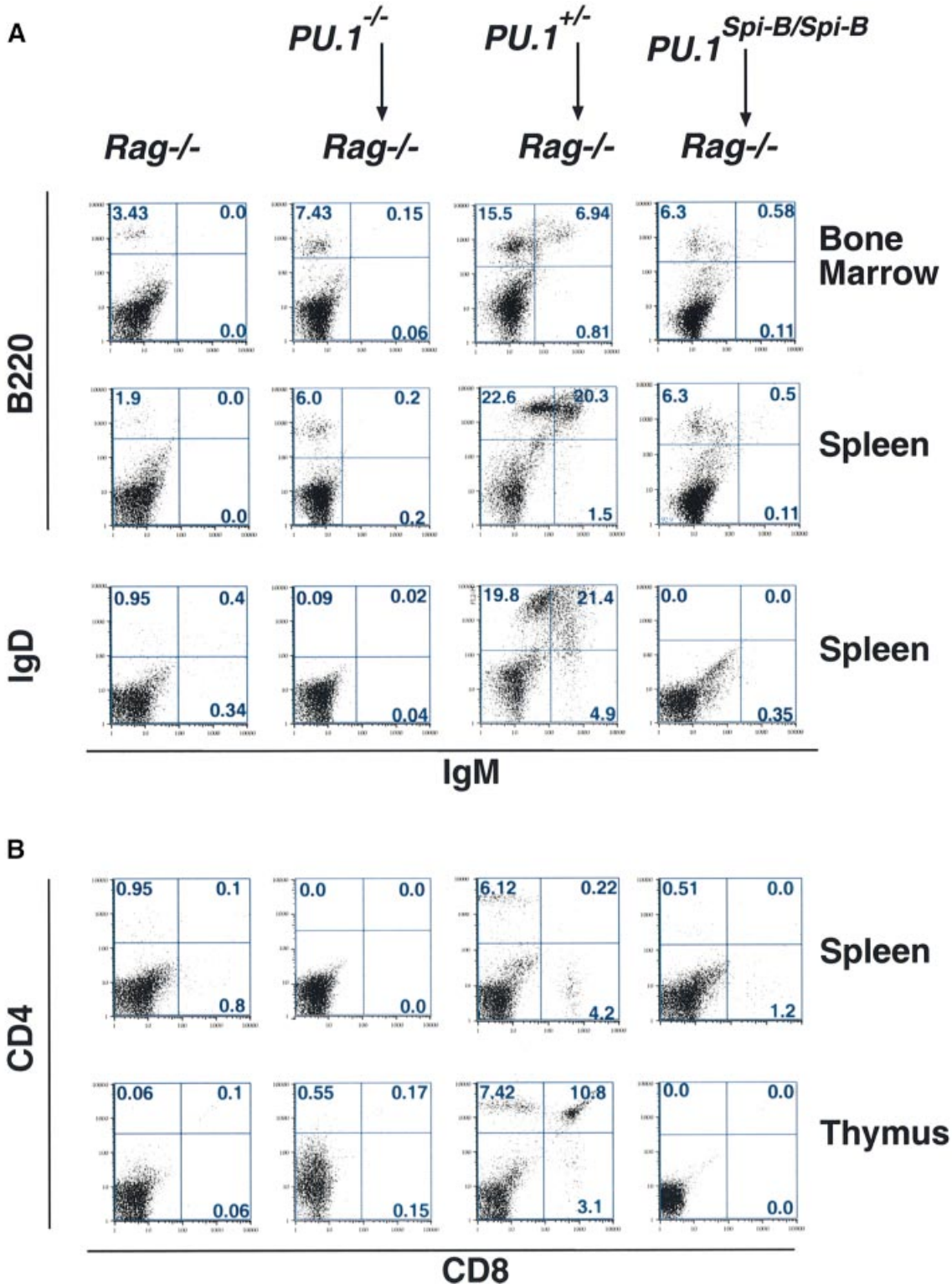

Fig. 8. Analysis of potential lymphoid rescue of *RAG-2*$^{-/-}$ mice. (A and B) *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$, *PU.1*$^{+/-}$or *PU.1*$^{-/-}$ ES cells were injected into *RAG-2*$^{-/-}$ blastocysts to produce control and experimental chimeras. Flow cytometric analysis of blood cell lineages within various tissues of chimeric mice was performed. This figure represents chimeric mice analyzed at 8 weeks of age. (**A**) Analysis of B220/IgM and IgM/IgD staining revealed no contribution to B-cell populations by *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ or *PU.1*$^{-/-}$ ES cells, while *PU.1*$^{+/-}$ contributed significantly to both bone marrow and splenic B cells. (**B**) Analysis of CD4/CD8 staining showed no contribution to either immature DP or mature SP T cells in the thymus or spleen of *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ or *PU.1*$^{-/-}$ chimeras, while *PU.1*$^{+/-}$ contributed to all populations.

ranging from 15 to 60% by both clones. Because various chimeric offspring appeared sickly at an early age, some mice were analyzed for lymphoid production at 2 or 4 weeks after birth. No chimeras analyzed at either 2, 4 or 8 weeks of age exhibited differentiated lymphoid cells. It is also noteworthy that multiple hematopoietic organs (e.g.

Table I. *RAG-2*$^{-/-}$ chimerism

ES cell genotype	% chimerism	Observations
PU$^{+/-}$	50	Analyzed at 6 weeks
	50	Analyzed at 6 weeks
	85	Analyzed at 8 weeks
	90	Analyzed at 8 weeks
	90	Analyzed at 8 weeks
	90	Analyzed at 8 weeks
PU$^{Spi\text{-}B/Spi\text{-}B}$	10[a]	Lethal
	15	Lethal
	30	Lethal
	15	Analyzed at 8 weeks
	25	Analyzed at 8 weeks
	25[a]	Analyzed at 4 weeks
	30	Analyzed at 4 weeks
	15	Analyzed at 4 weeks
PU$^{Spi\text{-}B/Spi\text{-}B}\Delta$neo	10	Analyzed at 8 weeks
	7	Analyzed at 8 weeks
	7	Analyzed at 8 weeks
	7	Analyzed at 8 weeks
	7	Analyzed at 8 weeks
	5	Analyzed at 8 weeks
	5	Analyzed at 8 weeks
	5	Analyzed at 8 weeks
	25[a]	Analyzed at 8 weeks
	30[a]	Analyzed at 2 weeks
	60[a]	Lethal
PU$^{-/-}$	95	Lethal
	30	Analyzed at 8 weeks
	30	Analyzed at 8 weeks
	60	Analyzed at 8 weeks

[a]Sickly.

bone marrow and spleen) exhibited a strong *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ ES cell contribution based on Southern blot assay (data not shown), but no mature lymphocytes were detected.

Regulation of lymphoid genes by Spi-B

We hypothesize that Spi-B cannot replace PU.1 in B-cell development due to an inability to regulate PU.1-dependent B-cell genes. We have shown previously that Spi-B binds poorly to PU.1 sites in the Ig J chain promoter, Igκ 3′ enhancer and Igλ$_{2-4}$ enhancer compared with PU.1 by gel shift analysis (Su *et al*., 1996; Rao *et al*., 1999b). We chose to examine the ability of Spi-B to transactivate reporter constructs containing the Igκ 3′ enhancer. The κ light chain is expressed in ~80% of murine B cells. PU.1 has been shown to cooperate with AP-1 and PIP to regulate the core Igκ 3′ enhancer in NIH-3T3 cells where the enhancer normally is silent (Pongubala and Atchison, 1997). We showed that PU.1 augments enhancer activity 6- to 7-fold over that induced by AP-1 and PIP alone (Figure 9). In direct contrast, Spi-B stimulated the Igκ enhancer by only 2- to 3-fold. Furthermore, other PU.1-regulated B-cell genes, such as *P2Y10* and c-*rel*, are transactivated weakly by Spi-B compared with PU.1 (Rao *et al*., 1999a; Hu *et al*., 2001). These results suggest that Spi-B is incapable of replacing PU.1 in B-cell development because it is does not have sufficient activity to regulate properly the critical lymphoid genes required for B-cell maturation.

Discussion

The Spi subfamily of Ets transcription factors is essential for normal hematopoiesis (Fisher and Scott, 1998; Simon,

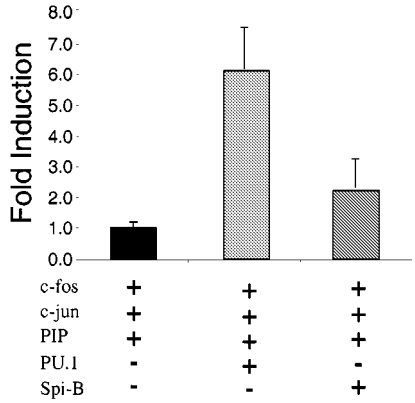

Fig. 9. Spi-B weakly transactivates the 3′ κ light chain enhancer. NIH 3T3 cells were transfected with the CoreLBKCAT and pGL2 control reporter plasmids along with plasmids expressing c-Jun, c-Fos and PIP. PU.1 and Spi-B expression plasmids were included in the transfections as indicated in the figure. Fold induction of CAT activity is relative to the transfection without PU.1 or Spi-B and is normalized to relative luciferase activity. Values obtained are an average of three independent transfections. Error bars represent standard deviations from the mean.

1998). To elucidate further the function of individual family members, we have investigated the redundancy of these proteins. We previously demonstrated a genetic interaction between Spi-B and PU.1. *PU.1*$^{+/-}$ *Spi-B*$^{-/-}$ mice exhibit a more severe B-cell receptor signaling defect than either the *Spi-B*$^{-/-}$ or *PU.1*$^{+/-}$ mutations alone. Presumably these defects are due to deregulation of target genes, and we have identified several genes underexpressed in the *PU.1*$^{+/-}$ *Spi-B*$^{-/-}$ mice (Rao *et al*., 1999a; Hu *et al*., 2001). However, our previous studies failed to distinguish if deregulation was due to a threshold effect for Spi family member activity or whether some target genes require the unique activities of each factor.

Other transcription factor families, including MyoD, GATA, Pax and Fos proteins, contain functionally redundant members based on gene knock-in approaches (Wang *et al*., 1996; Tsai *et al*., 1998; Bouchard *et al*., 2000; Fleischmann *et al*., 2000). In our study, gene targeting was used to introduce Spi-B and Ets-1 individually into the murine *PU.1* locus so that they would be expressed in place of PU.1. Spi-B is expressed predominantly in B lymphocytes, where it is co-expressed with PU.1 throughout most of B-cell development. Spi-B has never been detected in myeloid cells (Chen *et al*., 1995; Su *et al*., 1996). Ets-1 is broadly expressed and detected in most hematopoietic lineages, with high levels observed in adult lymphoid cells (Kola *et al*., 1993; Maroulakou *et al*., 1994). The present study allowed us to determine if these factors have evolved simply to provide Spi and/or Ets activity at the appropriate developmental time and place or if they have also evolved unique functional capabilities.

Our results demonstrate a requirement for Spi subfamily members in myeloid development, since Spi-B could rescue myeloid development but Ets-1 could not. In both primary EB differentiation and more sensitive hematopoietic progenitor assays, Spi-B, but not Ets-1, generated normal macrophages based on morphology and histological staining. Although we did observe a few *PU.1*$^{-/-}$ and *PU.1*$^{Ets\text{-}1/Ets\text{-}1}$ CFU-M colonies in methylcellulose cultures, histochemical analysis of these colonies did not reveal mature macrophages. Cells from such

colonies are potentially immature monocytes, in agreement with previous observations that PU.1 is not required for monocytic commitment but instead for maturation (Olson *et al*., 1995; DeKoter *et al*., 1998; Henkel *et al*., 1999). Along with inducing proper macrophage morphology, Spi-B activates myeloid-specific genes (CD11b and M-CSF receptor), which are poorly expressed in *PU.1*$^{-/-}$ cells. Spi-B-rescued cells were also able to phagocytose opsonized zymosan particles. The granulocytic lineage was also examined. From morphological analysis of CFU-G cells from hematopoietic colony assays, it appeared that Spi-B granulocytes were more mature than *PU.1*$^{-/-}$ cells, but not as mature as wild-type cells. This conclusion was corroborated by gene expression data that showed that Spi-B could rescue lysozyme, but not lactoferrin, expression.

Although Spi-B replaced PU.1 in myeloid development, it did not rescue B or T lymphopoiesis. Two independent ES cell clones shown to have myeloid activity failed to rescue lymphoid development in *RAG-2*$^{-/-}$ mice. B220^{+}/IgM^{+}, CD4^{+} SP, CD8^{+} SP and CD4^{+}/CD8^{+} DP cells were detected in chimeric mice generated with *PU.1*$^{+/-}$ ES cells, but not with *PU.1*$^{-/-}$ or *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ cells. Unfortunately, *PU.1*$^{PU.1/PU.1}$ ES cells could not be assayed since, to our surprise, they did not contribute significantly to any tissues in chimeric animals. Importantly, *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ cells contributed to the hematopoietic compartment as we detected the knock-in allele in splenic and bone marrow cells by Southern blot (data not shown). Since there was clear evidence of hematopoietic contribution, the lack of lymphoid development was not due to an inability to rescue the *PU.1*$^{-/-}$ bone marrow homing defect (Fisher *et al*., 1999).

It was surprising that Spi-B could rescue myeloid but not lymphoid development. Spi-B normally is expressed in B cells. Therefore, when expressed under the control of the *PU.1* locus, hSpi-B is in an environment that contains necessary co-factors for its normal function as a transcriptional regulator. One potential explanation for our results is that Spi-B protein or transcriptional activity is increased compared with endogenous PU.1. Levels of PU.1 activity have been shown to control cell fate decisions (DeKoter and Singh, 2000). Although this observation has yet to be demonstrated *in vivo*, it is possible that Spi-B protein levels and/or activity may be too high to direct the B-cell fate. We do not favor this interpretation. *In vitro* transcriptional assays so far have not detected a significantly increased transactivation ability of Spi-B compared with PU.1 (Ray *et al*., 1992; Ray-Gallet *et al*., 1995; Rao *et al*., 1999b). In addition, we show here that Spi-B transactivation of the Igκ 3′ enhancer is reduced compared with PU.1. We cannot directly compare levels of PU.1 and Spi-B due to different affinities of the respective antibodies. However, RT–PCR and western blot analysis of primary *PU.1*$^{+/Spi\text{-}B}$ B-cell RNA and protein do not indicate that the knocked-in *Spi-B* is overexpressed dramatically relative to endogenous PU.1 (Figure 2 and data not shown). Additionally, *PU.1*$^{+/Spi\text{–}B}$ mice produce normal numbers of B lymphocytes, indicating that the knocked-in Spi-B is not detrimental to lymphoid development (data not shown). Unfortunately, *PU.1*$^{Spi\text{-}B/Spi\text{-}B}$ mice die prior to embryonic day 11.5, precluding analysis of lymphopoiesis.

Our results show that Spi-B can replace PU.1 in myelopoiesis, but not lymphopoiesis. However, we cannot conclude that Spi-B replaces PU.1 in all aspects of myeloid cell function, and granulocytic rescue was shown to be incomplete. The functional differences we observe between PU.1 and Spi-B have important implications for the evolution of the Spi subfamily of Ets transcription factors. We previously described a threshold effect for PU.1 in B-cell function. Deleting one allele of *PU.1* in *Spi-B*$^{-/-}$ mice increases the severity of the BCR signaling defect. Genes critical for B-cell function are clearly dependent on Spi family members for proper expression. Expression of such genes is dependent either on the unique functional abilities of both factors or on a certain level of Spi activity. Our results suggest that genes involved in lymphoid lineage commitment and/or maturation are strictly dependent on PU.1 for their expression. We propose that B-cell lineage commitment exclusively requires PU.1, but proper B-cell function is dependent on both PU.1 and Spi-B.

Materials and methods

Generation of knock-in ES cells

To create a knock-in vector for targeting the murine *PU.1* locus, two genomic DNA fragments were isolated: a 2.3 kb *Sac*I fragment for 5′ homology and a 4.6 kb *Bam*HI fragment for 3′ homology. The 4.6 kb *Bam*HI fragment is downstream of *PU.1* exon 1, upstream of exon 3 and includes all of exon 2. The 4.6 kb fragment was inserted into the *Bam*HI site of targeting vector pLNT (pPNT with LoxP sites flanking the PGK::neo cassette). The 2.3 kb *Sac*I fragment is 6 bp upstream of the *PU.1* initiator codon within exon 1. This fragment was blunt ended and subcloned into pBluescript KS which had been digested with *Sac*II and blunt ended with T4 polymerase, creating the 5′ homology vector. hSpi-B, hEts-1 and mPU.1 cDNAs were amplified by PCR using primers, which engineered a 5′ flag epitope sequence with a *Sac*I site, and a 3′ primer with a *Sal*I site. These cDNAs were then subcloned into the 5′-homology vector. A 150 bp *Sal*I–*Xho*I fragment containing the SV40 poly(A) sequence was then inserted downstream of each cDNA. The 5′-homology arm, cDNA and poly(A) sequence were removed by *Not*I, *Xho*I digestion and ligated into the pLNT 3′-homology vector to generate the final targeting construct.

RW4 ES cells were electroporated with the targeting constructs and homologous recombinants were selected as previously described (Maltepe *et al*., 1997). The targeting results in a deletion of ~1 kb of genomic sequence from the *Sac*I site of exon 1 to a downstream intronic *Bam*HI site. Correctly targeted clones were identified by Southern blot analysis using a 350 bp *Ava*I–*Sac*I fragment from the *PU.1* genomic locus. To generate homozygous targeted cells, heterozygous clones were subjected to further selection in 2–4 mg/ml G418 (Mortensen *et al*., 1992).

Embryoid body generation and analysis

ES clones were *in vitro* differentiated into EBs for 11–16 days as previously described (Olson *et al*., 1995). EBs were harvested for cytocentrifugation onto glass microscope slides or disaggregated with trypsin and plated onto chamber slides (Nunc). Cytospin preparations were stained with May–Grunwald–Giemsa stain. Cultured EB-derived cell suspension was allowed to adhere for 24 h and then was washed to remove non-adherent cells. Adherent cells were then fixed and stained with rat anti-mouse F4/80 (Caltag) or anti-CD11b (Pharmingen), using a Vectastain ABC-alkaline phosphatase kit (Vector Laboratories Inc.).

Phagocytosis was examined by incubating adherent cells with opsonized FITC-labeled zymosan particles (Molecular Probes). Zymosan particles were opsonized by mixing equal volume of opsonizing reagent (Molecular Probes) with equal volume of zymosan particles and incubating at 37°C for 1 h. Zymosan particles were washed three times with phosphate-buffered saline (PBS) and resuspended in PBS/10% fetal calf serum/5 mM glucose. Approximately 1×10^6 particles were added to each well of cells and incubated for 30 min at 37°C. Cell cultures were washed extensively with PBS to remove unattached particles. Cells were

fixed in 3.7% formaldehyde and visualized with a Nikon Eclipse E800 microscope.

In vitro differentiation and replating of ES cells

ES cells were removed from leukemia-inhibiting factor (LIF)-containing media and plated in methylcellulose (Methocult 4100, Stem Cell Technologies) containing 10% serum, 500 U/ml rhIL-1, 5 ng/ml rmIL-3, 10 μg/ml insulin, 200 μg/ml transferrin and 10^{-4} M α-monothioglycerol, and allowed to differentiate at 37°C. After 9 days, EBs were harvested and disaggregated with trypsin and mechanical shearing with a 21 gauge needle. Cells were then replated into Methocult GF 3434 methylcellulose media (Stem Cell Technologies). The number of cells plated was equal to the number of cells in 50 EBs derived from wild-type ES cells. Hematopoietic colonies were scored and cytocentrifuged 5–7 days later.

RT–PCR analysis

RNA was isolated from hematopoietic progenitor cultures 7–9 days after replating primary EBs or from EB cultures differentiated for 11 days. Total cellular RNA was isolated using TRIzol (Gibco-BRL) according to the manufacturer's instructions. A 1 μg aliquot of total cellular RNA was used in a 20 μl reverse transcriptase reaction (Superscript II, Stratagene). A 2 μl aliquot of the reverse transcriptase reaction was used in a PCR with gene-specific primers. The primers used for amplification of knock-in cDNA were (1) GGATGGACTACAAGGACGACG and (2) GCTGCAATAAACAAGTTGG. Primers for M-CSFR, CD11b, HPRT, MPO, lactoferrin and lysozyme were described previously (Olson *et al*., 1995; Anderson *et al*., 1998). C/EBPε was amplified as previously described using primers RY48 and RY50 (Yamanaka *et al*., 1997a). Primer pairs did not generate appropriate size fragments from genomic DNA and/or from mock reverse-transcribed RNA preparations.

Enrichment of murine B lymphocytes

Spleens were isolated from $PU.1^{+/-}$ and $PU.1^{+/Spi\text{-}B}$ mice. Single-cell suspensions were generated and subjected to ACK lysis. The cell suspension was enriched for murine B lymphocytes using StemSep murine B-cell enrichment cocktail and StemSep magnetic columns according to the manufacturer's instructions (Stem Cell Technologies). Cells were analyzed by fluorescence-activated cell sorting (FACS) for expression of CD19 and B220 (Pharmingen).

Western blotting

EBs were isolated from methylcellulose, washed with PBS and directly lysed in SDS–PAGE sample buffer. U937 and clone 13 cells were also lysed in SDS–PAGE sample buffer; 500 000 cell equivalents were loaded per lane. Murine splenic B cells were lysed in RIPA buffer and total protein was precipitated with trichloroacetic acid. Approximately 150 μg of protein was loaded per lane. SDS–PAGE and western transfer were done using standard methods. Western blots were incubated with anti-PU.1 (Santa Cruz Biotechnology), anti-CREB (Cell Signaling Technology) or anti-hSpiB (Su *et al*., 1996).

RAG-2$^{-/-}$ complementation assay and FACS analysis

Chimeric mice were generated by injection of $PU.1^{+/-}$, $PU.1^{-/-}$ or $PU.1^{Spi\text{-}B/Spi\text{-}B}$ RW4 ES cells into $RAG\text{-}2^{-/-}$ blastocysts (Chen *et al*., 1993). The $RAG\text{-}2^{-/-}$ strain used had been bred for black coat color. The percentage chimerism was estimated by appearance of agouti coat color, which would be contributed by cells derived from the donor ES cells. Single-cell suspensions were prepared from bone marrow, spleens and thymi of chimeric animals. Cells were stained with FITC- or phycoerythrin (PE)-conjugated primary antibodies or with biotinylated primary antibodies, followed by streptavidin-labeled FITC, PE or cychrome. Stained cells were analyzed on a dual laser cell sorter (FACScan, Becton Dickinson). Cell preparations were pre-incubated with antibody to FcγRIII/II to reduce non-specific antibody binding and were subjected to propridium iodide uptake to exclude dead cells from the analysis. Monoclonal antibodies to B220, IgM, IgD, CD4 and CD8 were used according to the manufacturer's instructions (Pharmingen). FACS data were analyzed using FloJo software (Tree-Star).

Transient transfections

NIH 3T3 cells were transfected with the appropriate DNA using Superfect reagent according to the manufacturer's instructions (Qiagen). A 2 μg aliquot of reporter plasmid, CoreLBKCAT and 500 ng of one or more of the following plasmids was used per transfection: pCMV-jun, pCMV-fos, pCMV-PIP, pcDNA3-PU.1 and pcDNA3-Spi-B. A 500 ng aliquot of pGL2 control luciferase plasmid was used to normalize transfections. Total DNA content per transfection was brought up to 5 μg with pcDNA3.1. At 48 h post-transfection, cell extracts were prepared using Promega reporter lysis buffer. Chloramphenicol acetyl transferase (CAT) activity was measured using the Quan-T-CAT system (Amersham Life Science) and luciferase was measured using the Promega luciferase system.

Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

Acknowledgements

The authors are grateful to C.Clendenin, C.Culpepper and P.Thayer for generating chimeric mice. Michael Atchison generously provided us with the CoreLBKCAT plasmid. We would also like to acknowledge members of our laboratory for critically reading the manuscript. The NIH (grant 52094), the Abramson Family Cancer Research Institute and the Howard Hughes Medical Institute supported this work.

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Received June 25, 2001; revised February 1, 2002; accepted March 5, 2002

Note added in proof

While this work was under review, a paper entitled 'PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors' by D.P.DeKoter, H.J.Lee and H.Singh (*Immunity*, **16**, 297–309, 2002) was published. These authors demonstrate that retroviruses producing high levels of Spi-B generate $CD19^+$ B cells in *PU.1*$^{-/-}$ progenitor cultures. However, it should be noted that this *in vitro* system does not assess the production of $B220^+IgM^+$ B cells *in vivo* in bone marrow, or their transit and homing to secondary lymphoid organs, such as the spleen. Therefore, we conclude that Spi-B is unable to regulate IgM^+ B cell development in the absence of PU.1.