The EMBO Journal vol.15 no.20 pp.5647–5658, 1996

Targeted disruption of the *PU.1* gene results in multiple hematopoietic abnormalities

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PU.1 is a member of the ets family of transcription factors and is expressed exclusively in cells of the hematopoietic lineage. Mice homozygous for a disruption in the PU.1 DNA binding domain are born alive but die of severe septicemia within 48 h. The analysis of these neonates revealed a lack of mature macrophages, neutrophils, B cells and T cells, although erythrocytes and megakaryocytes were present. The absence of lymphoid commitment and development in null mice was not absolute, since mice maintained on antibiotics began to develop normal appearing T cells 3–5 days after birth. In contrast, mature B cells remained undetectable in these older mice. Within the myeloid lineage, despite a lack of macrophages in the older antibiotic-treated animals, a few cells with the characteristics of neutrophils began to appear by day 3. While the PU.1 protein appears not to be essential for myeloid and lymphoid lineage commitment, it is absolutely required for the normal differentiation of B cells and macrophages.

Keywords: ets family/hematopoiesis/PU.1/transcription factor

Introduction

Hematopoietic development is a dynamic but regulated process that begins in early embryogenesis and continues throughout adult life. The mature cells that arise during hematopoiesis are functionally and phenotypically distinct, although all lineages derive from common pluripotent hematopoietic stem cells (HSCs) (Wu *et al.*, 1967; Abramson *et al.*, 1977; Till and McCulloch, 1980; Dick *et al.*, 1985; Keller *et al.*, 1985; Lemischka *et al.*, 1986; reviewed in Orlic and Bodine, 1994). Based on *in vitro* and *in vivo* studies, it has been proposed that at least one pathway of non-lymphoid development from HSCs occurs via a multipotential precursor that gives rise to committed myeloid and/or erythroid progenitors (Abramson *et al.*, 1977). Demonstration of lymphoid lineage progenitors that arise from the HSCs and that can differentiate into either T, B or natural killer (NK) cells has been more difficult (reviewed in Lemischka, 1991; Phillips, 1991; Galy *et al.*, 1995). A major block to our understanding of HSC lineage commitment has been the paucity of suitable models to analyze hematopoietic progenitor–progeny relationships at the molecular and cellular level. Recent gene disruption experiments in animals have begun to contribute new insights to our understanding of hematopoietic lineage relationships and the role of specific transcription factors in regulating this process (reviewed in Pfeffer and Mak, 1994; Kehrl, 1995; Orkin, 1995; Shivdasani and Orkin, 1996). In particular, the targeted disruption of transcription factors in mice has demonstrated the critical importance of these factors in the commitment of cells to specific lineages.

PU.1 is a transcription factor that is expressed exclusively in hematopoietic cells and may be involved in lineage development. The *PU.1* (*Spi-1*, *Sfpi-1*) gene is a member of the ets family of transcription factors (reviewed in Janknecht and Nordheim, 1993; Moreau-Gachelin, 1994). Within the PU.1 protein is a DNA binding domain (ETS domain) of ~85 amino acids that is highly conserved among all ets family members and recognizes a purine-rich DNA sequence containing the core sequence 5′-GGAA/T-3′ (Karim *et al.*, 1990). This domain recently was crystallized, and the results indicate that PU.1 binds to DNA in a novel manner but has similarity to the winged helix–turn–helix family of proteins (Kodandapani *et al.*, 1996). Other domains within PU.1 include a glutamine-rich domain and acidic residues toward the N-terminal half of the protein that are necessary for transactivation (Klemsz and Maki, 1996), and a central PEST domain that is important for protein–protein interactions (Pongubala *et al.*, 1993).

Hematopoietic cells expressing PU.1 include B lymphocytes, macrophages, mast cells, neutrophils and early erythroblasts (Klemsz *et al.*, 1990; Chen *et al.*, 1993; Galson *et al.*, 1993; Henkel and Brown, 1994). While no expression of PU.1 has been observed in T cell lines, a low level of expression is seen in the mouse thymus (Klemsz *et al.*, 1990; Faust *et al.*, 1993; Hromas *et al.*, 1993). PU.1 has been linked to the regulation of a number of genes in both B cells and myeloid cells (reviewed in Moreau-Gachelin, 1994). It has also been shown to mediate differentiation of human multipotential progenitor $CD34^+$ cells (Voso *et al.*, 1994). Consistent with a role for PU.1 in hematopoiesis, Scott *et al.* (1994) recently demonstrated that insertional inactivation of the *PU.1* gene causes variable anemia and a lack of detectable mature B and T cells, granulocytes and macrophages. An additional and unexplained result from their study was that the inactivation of PU.1 led to embryonic lethality.

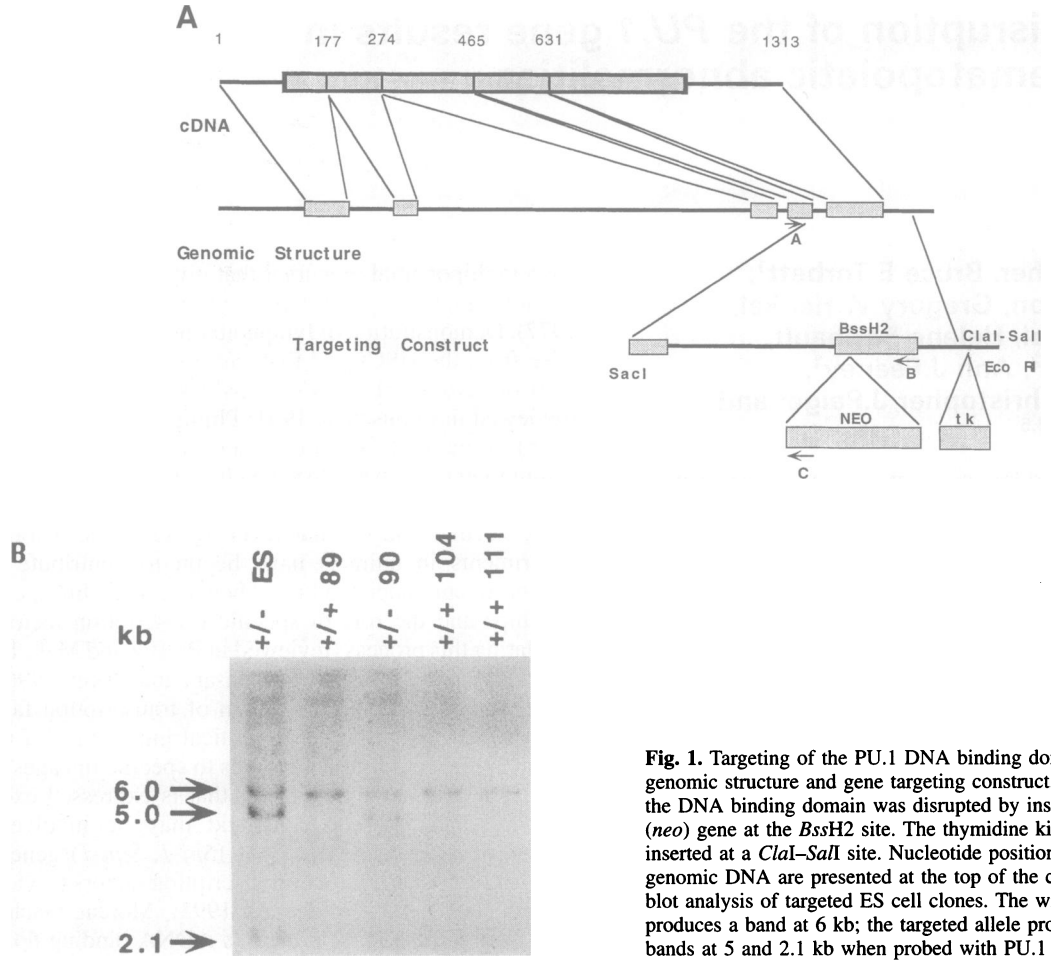

Fig. 1. Targeting of the PU.1 DNA binding domain. (**A**) PU.1 cDNA, genomic structure and gene targeting construct. The exon coding for the DNA binding domain was disrupted by insertion of the neomycin (*neo*) gene at the *Bss*H2 site. The thymidine kinase (*tk*) gene was inserted at a *Cla*I–*Sal*I site. Nucleotide positions of exons in the genomic DNA are presented at the top of the cDNA. (**B**) Southern blot analysis of targeted ES cell clones. The wild-type *PU.1* allele produces a band at 6 kb; the targeted allele produces hybridizable bands at 5 and 2.1 kb when probed with PU.1 cDNA.

In the present study, we find that mice homozygous for the disrupted *PU.1* gene are born with the expected Mendelian frequency, but develop fulminating septicemia and die within 48 h. At birth these mice are devoid of mature B and T lymphocytes, macrophages and neutrophils, but have erythrocytes and megakaryocytes. When the *PU.1* null mice are maintained on antibiotics, they can be kept alive for ~2 weeks after birth. During this time, T cell development is observed in the thymus, and $CD4^+$ and $CD8^+$ T cells begin to appear in the spleen. Although not present at birth in blood or hematopoietic tissues of null mice, cells with neutrophil characteristics begin to appear 3–5 days after birth. Mature B cells and macrophages, however, remain below the level of detection in these older mice. Based on these results, we believe that PU.1 plays a pivotal role in hematopoiesis.

Results

Generation of mice deficient in the expression of the PU.1 gene

The DNA binding domain (ETS domain) of PU.1 consists of a region of ~85 amino acids located near the C-terminal end of the protein. Disruption of the DNA binding domain of PU.1 should ablate its ability to bind DNA. To disrupt this domain, we designed a targeting construct using a double selection strategy previously developed (Mansour *et al.*, 1988). The exon coding for the DNA binding domain was disrupted by inserting the *neo* gene into a *Bss*H2 site within exon 5 of the *PU.1* gene (Figure 1A). Following electroporation of the targeting vector into D3 ES cells (Doetschman *et al.*, 1985), G418 and gancyclovir doubly resistant colonies were selected and screened by Southern blot analysis. For this analysis, genomic DNA was digested with *Eco*RI and probed with labeled PU.1 cDNA. The normal *PU.1* allele gave a single band of 6 kb, while the targeted allele gave two bands of 5 and 2.1 kb as expected (Figure 1B). Clones positive by Southern blot analysis were characterized further by PCR analysis of the genomic DNA using primers both outside (primer A, Figure 1) and inside (primers B and C, Figure 1) the targeting construct (data not shown). A number of clones shown to have targeted the *PU.1* gene were isolated. Three clones selected for injection into C57Bl/6 blastocysts gave rise to chimeric mice, but only one line displayed germline transmission. Mice heterozygous for the disrupted *PU.1* allele were phenotypically indistinguishable from their wild-type littermates and therefore are referred to as normal in this report.

An analysis of the genotypes of almost 200 offspring from the matings of heterozygous mice revealed the Mendelian ratio of homozygous *PU.1* null (25%), heterozygous (53%) and homozygous wild-type (22%) mice. The birth weights of *PU.1* null mice were not significantly different from the wild-type or heterozygous mice (data not shown). Their appearance was unremarkable, except for the occasional individual with a slightly darker red liver. The *PU.1* null mice all developed septicemia within

24 h and died by 48 h. With antibiotic treatment (enrofloxacin, 2.5 mg/kg), *PU.1* null mice have lived for up to 17 days.

Two lineages that appeared to be minimally affected in the neonatal *PU.1* null mice were the erythroid and megakaryocyte lineages. Examination of tissue sections and imprints of liver, spleen and bone marrow from *PU.1* null neonates revealed numerous erythroid cells in all stages of development (data not shown). Staining of hematopoietic tissues also revealed megakaryocytes, and peripheral blood smears showed platelets. These cell populations were not characterized further.

Macrophage development is absent in PU.1 null mice

Since PU.1 is expressed normally in monocytes/macrophages, we examined these cell types in the *PU.1* null mice by three different methods. First, tissue imprints from neonates or mice up to 15 days old were stained for non-specific esterase activity (Yam *et al.*, 1971). We observed many positive monocytes/macrophages in normal mice but none in the *PU.1* null mice. Second, immunohistochemical staining of multiple tissues (liver, spleen, thymus) from *PU.1* null neonates with a monoclonal antibody (F4/80) that identifies most macrophage populations (Austyn and Gordon, 1981; Hume *et al.*, 1983a; Morris *et al.*, 1991) failed to detect macrophages (Figure 2 and data not shown). In contrast, F4/80$^+$ cells were detected in all tissues examined from normal neonates (Figure 2 and data not shown). Interestingly, when the liver, spleen and thymus of 8- to 14-day-old *PU.1* null animals were examined, a small number of F4/80$^+$ cells were detected (Figure 2 and data not shown). The number of these cells in the *PU.1* null animals, however, was reduced drastically compared with normal littermates, and these F4/80$^+$ cells appeared abnormally large. This was particularly evident in the liver, where F4/80$^+$ cells from null mice were not only larger but also did not have the characteristic morphology or sinusoidal distribution of Kupffer cells, the predominant F4/80$^+$ cell normally found in this tissue. Flow cytometry using the F4/80 antibody corroborated the immunohistochemistry results of $<1\%$ F4/80$^+$ cells in bone marrow or liver from neonatal null mice. A similar result was obtained when bone marrow cells from antibiotic-treated 5- to 14-day-old animals were examined.

Since macrophages can be grown readily *in vitro* from normal animals in the presence of colony-stimulating factors, we established *in vitro* cell cultures from hematopoietic tissues to determine if macrophages could be grown *in vitro* from the *PU.1* null animals. Liver and spleen cell suspensions from fetal and neonatal normal or *PU.1* null mice were cultured in the presence of the growth factors macrophage colony-stimulating factor (M-CSF) or granulocyte–macrophage colony-stimulating factor (GM-CSF). Macrophages could be grown readily from normal mice but not from the *PU.1* null mice.

In summary, no mature macrophages were observed in the tissues of *PU.1* null neonates and only a few F4/80 staining cells were seen in the older *PU.1* null mice. This result, coupled with the lack of macrophage growth *in vitro* from fetal liver, leads us to conclude that the disruption of the *PU.1* gene inhibits normal macrophage development.

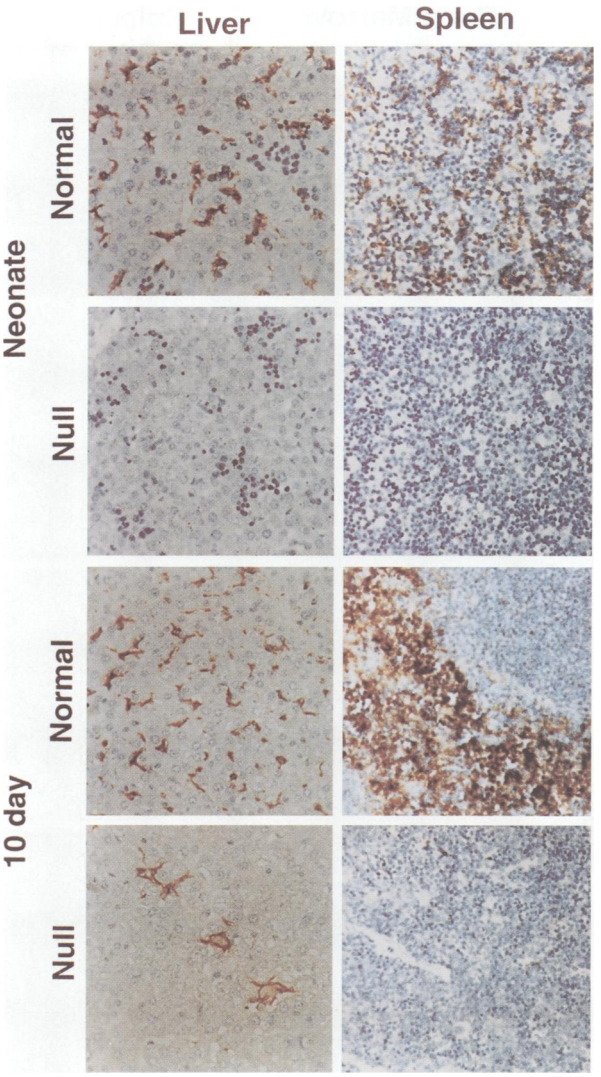

Fig. 2. Absence of F4/80$^+$ cells in *PU.1* null neonates and presence of a few aberrant F4/80$^+$ cells in older null mice. Shown are the liver and spleen of normal (+/+) and *PU.1* null (–/–) neonates and 10-day-old mice stained with F4/80 (500× magnification). Note the presence of numerous F4/80$^+$ cells in tissue from normal mice identifying monocytes/macrophages and resident macrophages/Kupffer cells, whereas in the *PU.1* null tissue note the paucity of F4/80$^+$ cells.

Neutrophil development is abnormal in PU.1 null mice

The fact that PU.1 has been shown to be expressed in neutrophils (Chen *et al.*, 1993) prompted us to investigate this lineage in the *PU.1* null mice. Since neutrophils have a characteristic nuclear morphology, tissue imprints were examined using Wright–Giemsa stain. No mature neutrophils were observed in blood, liver, spleen or bone marrow from *PU.1* null neonates, whereas these cells were numerous in comparable tissues from normal littermates. Cytochemical staining of tissue imprints for the neutrophil-specific enzyme chloroacetate esterase (CAE) was performed to detect less mature forms of neutrophils that might not be identifiable by routine Wright–Giemsa staining (Yam *et al.*, 1971). Whereas tissues of normal neonates contained many CAE-positive cells, comparable tissues of PU.1 null neonates were negative (Figure 3). By 3 days after birth, however, imprints of liver, spleen and bone

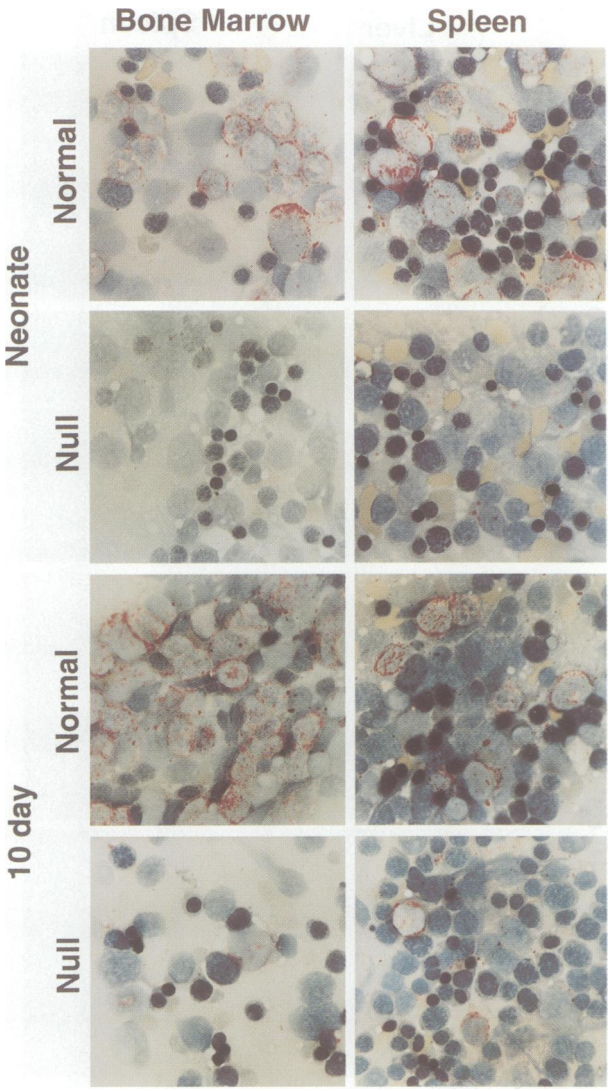

Fig. 3. CAE^+ neutrophils are absent at birth in *PU.1* null mice but appear within several days. Imprints of normal neonate and 10-day-old spleen compared with *PU.1* null neonate and 10-day-old spleen (at 1150× magnification). Note the presence of numerous erythroblasts and CAE^+ neutrophils in normal mouse tissue but only erythroblasts in the *PU.1* null neonate and rare CAE^+ cells in the 10-day-old mouse.

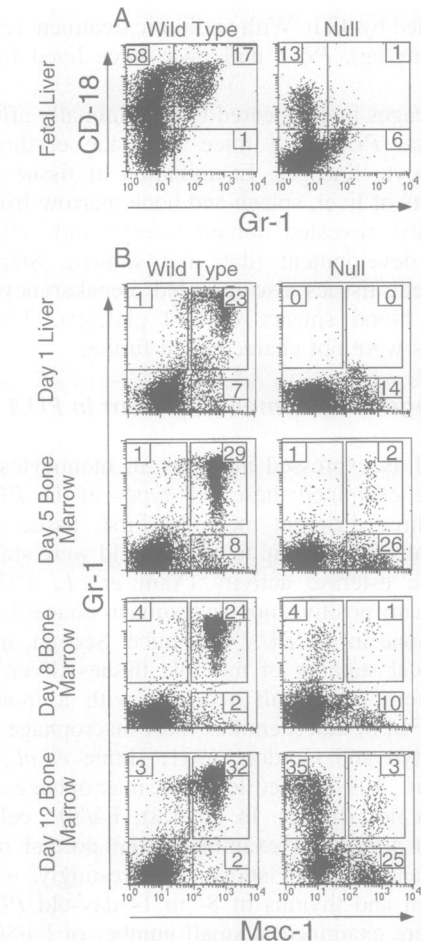

Fig. 4. Polymorphonuclear markers on cells from *PU.1* null liver or bone marrow are delayed and phenotypically aberrant. Liver or bone marrow cells from wild-type (+/+) or *PU.1* null (–/–) mice were analyzed for the presence of CD18 and Gr-1 in day 18 fetal liver (**A**), or Mac-1 and Gr-1 at 1, 5, 8 and 12 days after birth (**B**) using anti-$Mac\text{-}1^{FITC}$/anti-$Gr\text{-}1^{PE}$ mAbs. Percentages of CD18, Gr-1, CD18 and Gr-1, or Mac-1, Gr-1, Mac-1/Gr-1 and double-negative cells are indicated in each of the boxes for each sample. In (A) note the presence of $CD18^+$ cells and low numbers of $CD18^+/Gr\text{-}1^+$ cells. In (B) note the appearance over time of increasing numbers of $Gr\text{-}1^+/Mac\text{-}1^+$ cells (neutrophils) and of aberrant populations of $Gr\text{-}1^+/Mac\text{-}1^-$ and $Gr\text{-}1^-/Mac\text{-}1^+$ cells in the *PU.1* null mice.

marrow of null mice revealed a small number of morphologically mature CAE-positive neutrophils.

Although the tissue imprint studies demonstrated the presence of some neutrophils in *PU.1* null mice, we analyzed, by two-color flow cytometry, cell populations in liver and bone marrow in *PU.1* null mice to delineate further neutrophil development and neutrophil numbers. Antibodies were used that recognized CD18, Mac-1 (CD11b) and Gr-1, cell surface molecules that are associated with normal neutrophil development. CD18, a β2 integrin (reviewed in Hynes, 1987; Hemler, 1990), combines with various α chains and is expressed on many hematopoietic and lymphopoietic cell lineages. Mac-1 (CD11b), LFA-1 (CD11a) and gp150,95 (CD11c) are the different α chains using the common β chain, CD18. Gr-1 in combination with Mac-1 have been shown to be useful as phenotypic markers for neutrophil development. The expression of Gr-1 is negative/low on myeloblasts and increases as neutrophils mature, resulting in mature neutrophils expressing high levels of Gr-1/Mac-1 (Hestdal *et al.*, 1991). Gr-1 is found only transiently on cells of the monocytic lineage and not on mature macrophages (Hestdal *et al.*, 1991). Since CD18 is required for expression of all α chains and CD18 has been shown to have a PU.1 promoter site that appears to be required for expression (Rosmarin *et al.*, 1995), we first analyzed hematopoietic cells from day 18 fetal liver for the presence of CD18 and Gr-1 double-positive cells. Thus, we could address whether *PU.1* gene ablation disrupts expression of CD18 on developing neutrophils and determine the numbers of developing neutrophils.

As can be seen from results presented in Figure 4A, CD18 single-positive and CD18/Gr-1 double-positive cells were present in day 18 wild-type fetal liver. In contrast, fetal liver cells obtained from *PU.1* null mice of the same litter had a lower number of CD18 single-positive cells and 1% of the cells were CD18/Gr-1 double-positive. The

lack of mature neutrophils as shown by flow cytometry is consistent with the lack of neutrophils seen when neonatal liver was analyzed by immunohistochemical and cytochemical staining, as noted above. Thus, although CD18 can be expressed on cells when the *PU.1* gene is ablated, few if any CD18/Gr-1 double-positive cells were present, suggesting the absence of normal neutrophil development. Curiously, a $CD18^-/Gr\text{-}1^{-/lo}$ population was also noted.

To analyze further the time frame of neutrophil development in *PU.1* null mice, we used antibodies to Gr-1 and Mac-1. In this manner, we could determine if the population of developing $Gr\text{-}1^+$ neutrophils expressed cellular traits consistent with normal neutrophil development. Analyses by flow cytometry of liver cell suspensions from neonates and from bone marrow of 5-, 8- and 12-day-old *PU.1* null animals and their normal littermates revealed a large population of $Gr\text{-}1^+/Mac\text{-}1^+$ cells in normal animals at all ages, whereas in the *PU.1* null neonates there were few $Gr\text{-}1^+$ or $Gr\text{-}1^+/Mac\text{-}1^+$ cells (Figure 4). In contrast to the results found in *PU.1* null neonates, small numbers of $Gr\text{-}1^+/Mac\text{-}1^+$ cells began to appear in the liver and bone marrow of the *PU.1* null mice by 5 days, and single $Gr\text{-}1^+$ cells increased over time (Figure 4). Although few $Gr\text{-}1^+/Mac\text{-}1^+$ cells were observed in *PU.1* null mice, all of the $Gr\text{-}1^+$ cells were found to be $CD18^+$, demonstrating that the absence of Mac-1 expression on the majority of $Gr\text{-}1^+$ cells was most likely not due to the inability to express CD18 (data not shown). The levels of $Gr\text{-}1^+/Mac\text{-}1^+$ cells in the older null mice, however, never reached that observed in age-matched normal littermates. In addition to observing $Gr\text{-}1^+/Mac\text{-}1^-$, we saw a small population of $Gr\text{-}1^-/Mac\text{-}1^+$ cells in bone marrow (Figure 4) and spleen (data not shown) of older null animals. Consistent with the presence of $Gr\text{-}1^+$ cells, morphological, immunohistochemical and cytochemical staining of bone marrow and spleen of these older mice revealed cells with the characteristic morphology of neutrophils that were $Gr\text{-}1^+$ and CAE-positive. Thus, the development of $Gr\text{-}1^+$ cells was significantly delayed, and few $Gr\text{-}1^+/Mac\text{-}1^+$ double-positive neutrophils were observed.

Based on our results using a number of complementary approaches to delineate neutrophil development, we find that PU.1 is required for both the coordinated expression of Mac-1 and the timely development of neutrophils. Furthermore, a $Gr\text{-}1^+/Mac\text{-}1^-$ population with a neutrophil morphology seen in the *PU.1* null mice may represent atypical myeloid/neutrophil development. The exact relationship between the $Gr\text{-}1^+/Mac\text{-}1^+$, $Gr\text{-}1^-/Mac\text{-}1^+$ and $Gr\text{-}1^+/Mac\text{-}1^-$ cells and mature CAE-positive neutrophils in *PU.1* null mice is not clearly understood and is currently under investigation.

B cell differentiation is aberrant and does not proceed beyond an early stage in the PU.1 null mice

The histological architecture of the spleen of *PU.1* null neonates was indistinguishable from that of normal littermates. At birth, this organ contains a mixture of hematopoietic cells but, by 12 days of age in normal mice, splenic morphology is well defined with distinct red and white pulp. In contrast, the spleen of 12 day old *PU.1* null mice shows only minimal white pulp development, consistent with the presence of T cells (see below).

Given the apparent lack of normal splenic development and the fact that PU.1 is normally expressed in B cells, we examined whether B cell populations were altered in the *PU.1* null mice. Flow cytometry was used to analyze the presence of B cell progenitors using the markers immunoglobulin μ, B220, CD43, BP1 and HSA (Figure 5). Several significant differences between normal mice and the *PU.1* null littermates were observed. During normal B cell development, the expression of B220 continues to increase, while a decrease and eventual loss of CD43 expression takes place (Hardy *et al.*, 1991). This is accompanied by the sequential expression of HSA and BP1 followed by the loss of these markers as cells differentiate to mature B cells (Hardy *et al.*, 1991). Based on the expression of these markers, populations of $B220^+$ cells can be found in the bone marrow and spleen of normal neonates with the expected surface characteristics of pro-B, pre-B, immature B and mature B cells. In contrast, the $B220^+$ cells found in the *PU.1* null neonates are nearly all $CD43^-$, $BP\text{-}1^+$, $HSA^{lo/-}$ cells. These data confirm that cells bearing B lineage markers are present in 1-day-old bone marrow and spleen but that the pattern of differentiation differs from normal mice. Moreover, the predominant population does not correspond to a previously recognized phenotype. Similar results were seen in the older antibiotic-treated *PU.1* null mice. These results suggest that a significant alteration in the normal developmental pathway of the B lineage has occurred in the *PU.1* null mice.

RT–PCR analysis was used to test for the expression of λ5, a gene expressed in early B lymphocyte development, Rag 1 and Rag 2, both of which are necessary for immunoglobulin gene rearrangement, and mu°, whose expression precedes the rearrangement of heavy chain genes. In addition, we looked for evidence of immunoglobulin heavy chain gene rearrangement, both D–J and V–D–J, in the *PU.1* null animals (Bain *et al.*, 1995; Sollbach and Wu, 1995; Marshall *et al.*, 1996). We detected expression of rag 1, rag 2 and mu° but not λ5, in the *PU.1* null fetal liver. In the 10-day-old bone marrow, both Rag 1 and Rag 2 expression was detected but not mu° or λ5. In addition, we did not observe any evidence for heavy chain rearrangement in either the fetal liver or 10-day-old bone marrow of the *PU.1* null animals. However, expression of the respective genes along with rearrangement of immunoglobulin heavy chain genes was found in normal littermates.

Based on these data, we conclude that the *PU.1* gene is necessary for the maturation of B lymphocytes. Rather than a defined block in B cell development with the accumulation of normal progenitors, however, it appears that the lack of PU.1 results in the loss of coordinate expression of B lineage traits.

T cell differentiation is delayed in PU.1 null mice

Given our observation that hematopoietic cells in the *PU.1* null mice commit to the B cell pathway, but do not differentiate into mature B cells, it was of interest to determine if T cell development was affected by the loss of PU.1. Whether PU.1 has any direct role in T cell development is not known. Although PU.1 has not been detected in T cell lines, it is expressed at a low level in the thymus (Klemsz *et al.*, 1990; Hromas *et al.*, 1993).

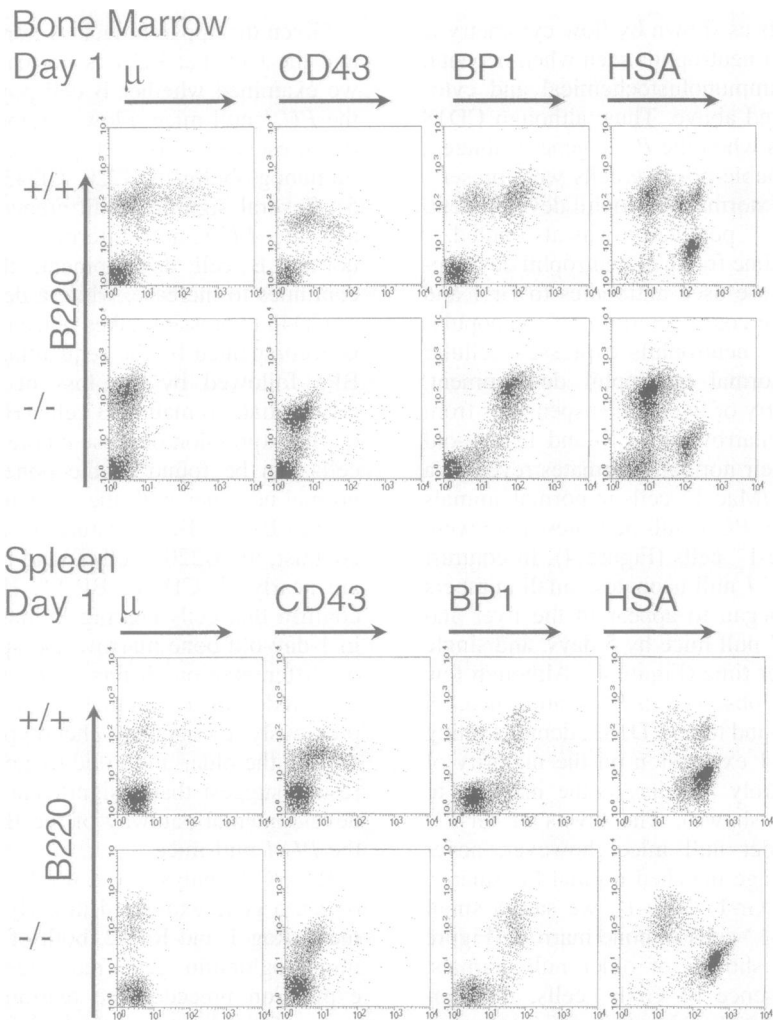

Fig. 5. Expression pattern of cell surface markers reveals that B cell development in *PU.1* null mice is abnormal. Bone marrow (day 1) and spleen (day 1) from wild-type (+/+) and *PU.1* null (–/–) mice were analyzed for the presence of μ, CD43, BP1, HSA and B220 by flow cytometry.

Macroscopic examination of thymi from *PU.1* null neonates revealed this organ to be about one-tenth of normal size, and microscopic examination revealed hypocellularity with no evidence of an organized cortex and medulla. Thymic cell suspensions were analyzed for selected T cell markers by flow cytometry. No T cell receptor (TCR) α/β^+, $CD3^+$, $CD4^+$ or $CD8^+$ thymocytes were detected from *PU.1* null neonates by flow cytometry (Figure 6 and data not shown). In addition, in the neonatal *PU.1* null mice, the total cell yield from each thymus was $<10^4$ cells. In contrast, all normal littermates had detectable $CD4^+$ and $CD8^+$ thymocytes (Figure 6).

Interestingly, by 5 days of age, $CD4^+$ and $CD8^+$ thymocytes appeared in some antibiotic-treated *PU.1* null mice (Figure 6), and by day 8 all antibiotic-treated *PU.1* null mice had $CD4^+$ and $CD8^+$ single- and double-positive thymocytes. The total numbers of thymocytes present in *PU.1* null mice up to day 12 were at least 5- to 10-fold lower than in normal littermates. Histological examination of thymi from these older *PU.1* null mice demonstrated both defined cortical and medullary regions. We also noted differences in the CD4/CD8 ratios in cells from the thymi of *PU.1* null mice, perhaps reflecting aberrant T cell selection due to a direct effect of PU.1 or due to an abnormal thymic microenvironment. Both the double-positive CD4/CD8 and single-positive CD4 and CD8 T cells expressed TCR α/β/CD3. Emigration of mature $CD4^+$ and $CD8^+$ cells from the thymus to the periphery was evidenced by the detection of low numbers (1–10%) of these cells in the spleen of the *PU.1* null mice by day 8. Interestingly, although double-positive CD4/CD8 T cells were present in the spleen by day 8, the white pulp areas of the spleen were very underdeveloped, probably reflecting the lack of B cells and macrophages and the low number of double-positive CD4/CD8 T cells. We have not yet determined the status of γ/δ T cells, dendritic or NK cells in these mice.

Thus, the absence of PU.1 does not appear to preclude B and T lymphoid commitment but does affect B and T cell development very differently. The lack of PU.1 resulted in the loss of sequential and coordinate expression of B lineage markers and a subsequent failure to differentiate to mature B cells. Although delayed and reduced in number, mature $CD4^+$ and $CD8^+$ T cells were observed in the thymus and periphery.

Discussion

Here we describe the disruption of the *PU.1* gene and its effects on hematopoietic development in the mouse (Table

I). At birth, animals born with this mutation lack mature macrophages, neutrophils, B cells and T cells. Subsequently, the *PU.1* null animals develop low numbers of mature T cells and cells resembling neutrophils. No apparent effect of this mutation was seen on the maturation of erythroid cells and megakaryocytes. Thus, the *PU.1* gene is apparently not essential for myeloid and lymphoid lineage commitment, but the gene is required for normal B cell, neutrophil and monocyte/macrophage lineage development. This pattern of PU.1 hematopoietic disruption is consistent with a lineage-specific effect. However, there may be indirect effects of this mutation as well, since there were delays in the appearance of $CD4^+$ and $CD8^+$ T cells, a lineage that normally does not express PU.1.

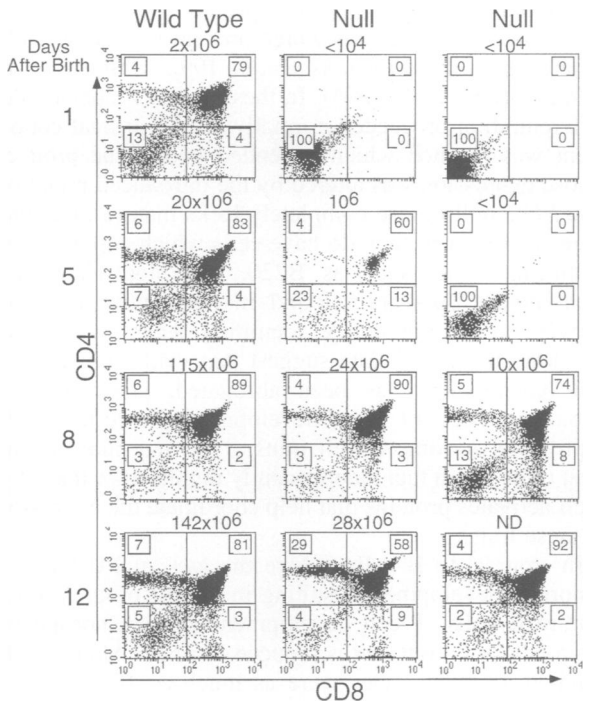

Fig. 6. T cell development in PU.1 null thymus is delayed and total number of thymocytes is reduced. Thymocytes from wild-type (+/+) or *PU.1* null (–/–) mice were analyzed at 1, 5, 8 and 12 days after birth using anti-$CD4^{PE}$ and anti-$CD8^{FITC}$ monoclonal antibodies. Percentages of $CD4^+$, $CD8^+$, double-positive and double-negative thymocytes are indicated in each quadrant for each sample. Total numbers of cells are indicated at the top of each analysis plot. Note the delay in thymocyte development and the reduced numbers of thymocytes at day 12 as compared with wild-type control. Also note the variability in thymocyte development in *PU.1* null littermates. ND, not done.

A possible role for PU.1 in regulating myeloid cell development was anticipated based on its expression in macrophages, mast cells, neutrophils, microglia and osteoclasts (Klemsz *et al.*, 1990; Henkel and Brown, 1994; Chen *et al.*, 1995; M.Tondravi and R.Maki, unpublished results). The absence of mature macrophages in the *PU.1* null mice strongly suggests that PU.1 is important for their development. Macrophages are one of the earliest recognizable hematopoietic cell types present in the embryonic yolk sac at 9–10 days post-coitum in the mouse (Cline and Moore, 1972; reviewed in Metcalf and Moore, 1971). $F4/80^+$ cells are first detected in the embryonic day 9–10 yolk sac; shortly thereafter they are found in the liver and other embryonic tissues (Morris *et al.*, 1991). The lack of $F4/80^+$ cells in embryonic day 10 yolk sac (K.Anderson and R.Maki, unpublished results.), 15 day fetal liver or in any neonatal tissue from the *PU.1* null mice, strongly suggests that the block in macrophage development occurs early in gestation. The finding of a few $F4/80^+$ cells which are larger than normal macrophages in the older *PU.1* null animals suggests that development of myeloid cells along the macrophage pathway can occur, but at a very low frequency. Whether the few $F4/80^+$ cells that develop in the *PU.1* null animals have any functional capabilities related to normal macrophages is not known.

Neutrophil development is also affected by the disruption of the *PU.1* gene. Whereas in normal mice mature neutrophils could be detected as early as embryonic day 11–12, in the *PU.1* null mice low levels of cells with characteristics of neutrophils were not seen until 3–5 days after birth. The presence of a $Gr\text{-}1^+$ population of cells in *PU.1* null mice demonstrates that the absence of PU.1 does not preclude neutrophil lineage commitment. The $Gr\text{-}1^{-/lo}$ population has been reported to contain the progenitors for both granulocytes and macrophages, whereas the Gr-1 bright cells predominantly consist of mature differentiated granulocytes (Hestdal *et al.*, 1991). Although the number of $Gr\text{-}1^+$ cells increased over time in the antibiotic-treated *PU.1* null mice, the number of cells bearing the normal neutrophilic $Gr\text{-}1^+/Mac\text{-}1^+$ phenotype increased only slightly (up to 4%). These results suggest that only a few $Gr\text{-}1^+$ cells are progressing to neutrophils expressing $Mac\text{-}1^+$. The presence of a population of $Gr\text{-}1^+$ cells, that were also CAE^+ and had the nuclear morphology of neutrophils, suggests that a few myeloid cells in the

Table I. There are multiple hematopoietic defects in *PU.1* null mice

Cell type	Age of *PU.1* null mice			Comments
	E15	Day 1	Day 10	
Erythrocytes	+	+	+	appear normal
Megakaryocytes	+	+	+	appear normal
Macrophages	–	–	–	a few F4/80 staining cells begin to appear at day 10 but they are abnormal in size and shape
Neutrophils	–	–	+	no mature neutrophils, cells with neutrophil morphology begin to appear soon after birth, CAE positive, $Gr\text{-}1^+/Mac\text{-}1^-$
B cells	–	–	–	no mature B cells, appearance of B220-, BP1- and CD43-positive cells but not in a previously described pattern
T cells	–	–	+	$CD4^+/CD8^+$ cells begin to appear soon after birth, TCR^+

Animals were sacrificed on embryonic day 15 (E15) or post-natal day 1 or 10. The presence (+) or absence (–) of specific lineages was examined.

PU.1 null mice are able to develop into cells with characteristics of neutrophils.

The absence of PU.1 could be critical for the expression of Mac-1 during neutrophil development, given the presence of PU.1 sites within the promoter of CD11b (Pahl *et al.*, 1993) and CD18 (Rosmarin *et al.*, 1995). Regulation of Mac-1 and CD18 are both reported to be PU.1 dependent (Pahl *et al.*, 1993; Rosmarin *et al.*, 1995), but both are found on hematopoietic cells in the *PU.1* null mouse. Therefore, it appears that expression of both Mac-1 and CD18 can occur in the *PU.1* null mouse and thus their expression may be complemented by other transcription factors. Although *PU.1* null hematopoietic cells expressed both CD18 and Mac-1, and Gr-1$^+$ myeloid cells expressed CD18, few Gr-1$^+$ cells were Mac-1 positive. Various adhesion molecules have been postulated to play an integral part in hematopoietic cell migration (Hirsch *et al.*, 1996), neutrophil differentiation (Lund-Johansen and Terstappen, 1993) and function (reviewed in Springer *et al.*, 1987; Springer, 1994). Thus, it is tempting to speculate that a possible explanation for abnormal neutrophil development and the septicemia observed in the *PU.1* null mice is that the disruption of the *PU.1* gene results in the loss of or in the inappropriate expression of cell surface adhesion molecules, such as Mac-1, that are needed for development, migration and function.

The disruption of normal myeloid development in *PU.1* null mice could be explained at least partially by the inappropriate expression or absence of growth factors, their receptors and/or the inability of myeloid cells to respond to required growth factors. Recent studies have suggested that PU.1 is critical in regulation of the expression of M-CSF (Zhang *et al.*, 1994), GM-CSF (Hohaus *et al.*, 1995) and G-CSF (Smith *et al.*, 1996) receptors. To our knowledge, since mice containing a disruption of all three of the above cytokine receptors have not yet been generated, the resulting effect on myeloid development remains unknown. While we do not believe that PU.1 directly regulates the expression of the M-CSF, GM-CSF or G-CSF cytokines, the disruption of these cytokine genes in mice provides a useful model to compare with the potential lack of expression of the corresponding receptors in the *PU.1* null mouse. In a preliminary study, disruption of GM-CSF, G-CSF and M-CSF cytokine expression did not ablate development of macrophages and neutrophils, although the levels of cells were decreased (Seymour *et al.*, 1995). This study suggests that cytokines other than GM-CSF, G-CSF and M-CSF are capable of supporting myeloid development *in vivo*. Whether the potential dysfunction or absence of GM-CSF, G-CSF and M-CSF receptors in the *PU.1* null mouse accounts for both the lack of macrophages and the neutrophil abnormalities remains to be determined. It is tempting, however, to speculate, based on the above-mentioned mouse studies, that loss of PU.1 results in the dysregulation of gene or cellular functions that contribute to the observed myeloid phenotype independently of the potential loss of GM-CSF, G-CSF and M-CSF receptors. Interestingly, mRNAs for both the M-CSF and GM-CSF receptors were detected in fetal liver and spleen by RT–PCR (unpublished results). It is possible, however, that our observation that mRNA for these receptors exists in the *PU.1* null mouse results from the expression of these receptors on other cell types or expression at an inappropriate time for normal myeloid development. Finally, our analysis of myeloid development in *PU.1* null mice suggests that monocytes/macrophages are absent and neutrophil commitment takes place, but that neutrophil development is abnormal and expansion occurs poorly at best.

Although the progression along the B cell pathway has been well characterized based on differentiation markers, growth factor responsiveness and immunoglobulin gene rearrangement, it is not certain to what extent these traits are dependent on each other. The generation of mice lacking particular gene products associated with B cell development has proven informative in this regard. In several instances, a block in B cell differentiation at the pro-B (B220$^+$, CD34$^+$, μ^+) stage has been observed in mice that carry mutations which prevent immunoglobulin gene expression (Mobaerts *et al.*, 1992; Shinkai *et al.*, 1992; Loffert *et al.*, 1994). In these cases, it appears that differentiation proceeded normally until a critical control point was reached which depended upon gene products whose expression was altered by the introduced mutation. The *PU.1* null mouse completely lacks mature functional B cells. However, they do have cells which express some B lineage traits such as B220, BP1 and HSA. The predominant population found in these mice does not correspond to previously identified stages of B cell development. These data suggest that while functional B cell development has been abrogated, progenitors do initiate a process of B cell development that results in the expression of some B cell traits. This may indicate that such traits are in fact independently regulated or that PU.1 itself activates proteins that help coordinate the expression of these traits.

In contrast to B cells, where the lack of PU.1 causes abnormal development resulting in loss of progression to mature B cells, T cell development appears normal but has a delayed onset and is reduced in scale. Although the numbers of thymocytes were as much as 10-fold lower than in wild-type littermates, the thymi from *PU.1* null mice demonstrated detectable cortical and medullary regions, and mature CD4$^+$ and CD8$^+$ T cells, traits consistent with normal T cell development. T cells also emigrated from the thymus to the periphery, consistent with normal T cell migration. In wild-type mice, T cell development commences around embryonic day 14 (Penit and Vasseur, 1989), whereas T cell development in the thymi of *PU.1* null mice did not begin until day 4–5 after birth (Figure 6). Whether this reduction in T cell number is a direct effect of PU.1 on early T cell progenitor development, a reduction in general hematopoietic development caused by a loss of PU.1 thereby delaying thymic progenitor seeding, or a reduction in thymic stromal support of thymic progenitors (Rodewald *et al.*, 1995) has not been determined.

The presence and expansion of TCR α/β-bearing thymocyte populations in the *PU.1* null mouse thymus are consistent with ongoing positive selection (reviewed in Jameson *et al.*, 1995) and expansion (Moore and Zlotik, 1995); whether negative selection is functioning in these mice remains to be determined. Although macrophages have been shown to play a role in negative selection of T cells and removal of apoptotic cells in the thymus (Surh and Sprent, 1994), the absence of macrophages appears

not to halt T cell development in the *PU.1* null mice. Additionally, the presence of defined cortical and medullary regions in the thymus and observed T cell expansion in the *PU.1* null mouse are consistent with the view that macrophages are not necessary for thymic organization or expansion.

An equally exciting aspect of the disruption of the *PU.1* gene was the finding that, despite the lack of macrophages, the *PU.1* null mice were born alive. Macrophages have been thought to play an important role during embryonic development in almost every tissue. For example, macrophages appear in large numbers synchronously with the large scale cell death occurring during tissue remodeling, including the regression of interdigital tissues (Hopkinson-Woolley *et al.*, 1994), chick wing bud posterior necrotic zone (Saunders, 1966), amphibian tail (Weber, 1964) and cell death within the central nervous system (Hume *et al.*, 1983b; Young 1984; Ferrer *et al.*, 1990). A causative, rather than simply a consequential, role for macrophages in cell death has been indicated in the process of pupillary membrane and hyaloid vasculature regression (Lang and Bishop, 1993; Lang *et al.*, 1994), raising the possibility that such an active phenomenon may occur elsewhere in development. Other than the hematopoietic deficiencies, we have detected no gross histological abnormalities in the *PU.1* null mice, suggesting that macrophages are not essential for development although not all tissues have been examined rigorously.

Besides playing an integral role in inflammation and immunity, macrophages are secretory cells that release a wide variety of factors important for cell growth and development (reviewed in Crocker and Milon, 1992; Rappolee and Werb, 1992). For instance, cells identified as macrophages produce erythropoietin in the fetal liver (Gruber *et al.*, 1977) and associate with developing erythroid cells in fetal liver and bone marrow. Macrophages have also been reported to be involved in the development of myeloid cells (Crocker and Gordon, 1989) and megakaryocytes (Crocker and Milon, 1992). The lack of macrophages in the *PU.1* null mouse and the subsequent lack of the appropriate growth or differentiation factors may have an adverse effect on hematopoiesis and thus may provide one explanation for the delay seen in the development of neutrophils and T cells.

A comparison of the *PU.1* null mouse discussed here and that generated by Scott *et al.* (1994) reveals many similarities but also some major differences. One major difference is that the *PU.1* null mice reported here were born alive while the *PU.1* null mice reported by Scott *et al.* (1994) died at embryonic day 18. A second major difference is that the defect in the *PU.1* null mice reported by Scott *et al.* (1994) results in a block in early development of both the myeloid and lymphoid lineages (Singh, 1996). In the mice reported here, there is both myeloid and lymphoid lineage commitment, but mature macrophages and B cells are absent. We do see the development of mature T cells and cells that resemble neutrophils, albeit that the appearance of these lineages is delayed.

There are several possible explanations for these interesting differences in mice generated by Scott *et al.* (1994) and the mice reported here. First, the constructs made by the two groups differ in that the insertion of the *neo* gene by Scott *et al.* (1994) was 35 amino acids upstream of the insertion site we used, and they also deleted the remainder of the exon containing the DNA binding domain. Furthermore, the orientation of the *neo* gene in the knockout developed by Scott *et al.* (1994) was in the same orientation as the *PU.1* gene and utilized the strong promoter from PGK, while the *neo* gene in our construct was in the opposite orientation to the *PU.1* gene and used the relatively weaker promoter from HSV-*tk*. The choice of the insertion site for gene disruption in the targeted gene as well as the orientation of the *neo* gene and its promoter has been shown recently to be critical for the resulting phenotype of the mouse (reviewed in Olson *et al.*, 1996). Recent studies in which the myogenic basic helix–loop–helix gene *MRF4/herculin/myf6* was disrupted to produce a null allele by three different groups resulted in three distinct phenotypes, with some shared similarities, ranging in severity from mild to lethal (reviewed in Olson *et al.*, 1996). The most likely explanation for these differences is that, in addition to disrupting *MRF4* gene expression, the investigators affected the expression to varying degrees of the nearby gene, *Myf5*, which is also a myogenic regulatory factor. This possibility must also be taken into account when considering the differences between the *PU.1* null mouse generated by Scott *et al.* (1994) and the *PU.1* null mouse reported here.

A second possibility for the differences is that a read-through transcript gives rise to a partial protein. However, we have tested nuclear extracts prepared from our *PU.1* null mice in a gel electrophoresis DNA binding assay using a consensus PU.1 DNA binding site and have found no evidence for a PU.1–DNA complex. Furthermore, structural analysis as well as mutational studies suggest that a disruption of the DNA binding domain such as was made in the construct reported here would make it very unlikely that the protein could still bind DNA (Kodandapani *et al.*, 1996). Third, while the strain of mice has been shown recently to influence the phenotype of a knockout (Baribault *et al.*, 1994; Threadgill *et al.*, 1995; Sibilia and Wagner, 1995), we do not believe that this can account for the differences in the phenotypes between the two groups since both groups characterized 129SV×C57Bl/6 mice. Other possibilities including differences in the embryonic stem (ES) cells used will require further investigation.

The disruption of the DNA binding domain of PU.1 leads to a profound effect on development within the hematopoietic system. It would appear from our *in vivo* and *in vitro* studies that the lack of functional PU.1 protein does not affect commitment of HSCs to the myeloid and lymphoid lineages but does affect the further development to specific cell types. The *PU.1* null mouse should provide an excellent model system for future studies to address the molecular role of PU.1 in the development of specific cell lineages within the hematopoietic system. Commitment of HSCs in the *PU.1* null mouse to either the myeloid or lymphoid lineage should permit us to address the stage at which specific lineage development cannot proceed and to identify those genes that play a role downstream of *PU.1*. In addition, the lack of tissue macrophages including Kupffer cells, alveolar macrophages and microglia makes this potentially an interesting

model to address the role of macrophages in tissue remodeling and in disease.

Materials and methods

Gene targeting construct and homologous recombination in ES cells

A 6 kb *Eco*RI genomic fragment in pBluescript KS$^+$ (Stratagene, La Jolla, CA) containing the third, fourth and fifth exons of the murine *PU.1* gene was used as the source for the targeting construct. This construct, described in Figure 1A, was created by excision of two *Sac*I fragments totalling 3 kb, leaving 3 kb containing part of the fourth and all of the fifth exons and part of the 3′ untranslated region of the *PU.1* gene. The HSV-*tk*-*neo* gene (1.2 kb) from pMC1neopolyA (Thomas and Capecchi, 1987) was blunt-end ligated into the *Bss*H2 site in the fifth exon in the antisense direction. The HSV-*tk* gene from pIC-1gR/MC1.tk (Mansour *et al.*, 1988) was inserted into the *Cla*I–*Sal*I sites of the vector at the 3′ end, and the *Hin*dIII site in the polycloning region of the vector was ablated. This arrangement made 0.8 kb 5′ and 2.2 kb 3′ of the *neo* gene insert available for homologous recombination. Electroporation was done using a BTX Transfector 100 power source set at 250 V and 5 ms. The targeting construct was linearized with *Hin*dIII and 40 μg of vector was electroporated into 5×10^7 cells. The recipient cells were D3jm p47 ES cells (Doetschman *et al.*, 1985) maintained on embryonic fibroblast feeder cells. G418 (150 μg/ml) and gancyclovir (300 μg/ml)-resistant colonies were picked and grown in 35 mm dishes. Genomic DNA was isolated from the cells, digested with *Eco*RI and analyzed by Southern blot using the PU.1 cDNA as probe. Positive clones were assayed further by Southern blot using genomic DNA digested with the alternative restriction enzymes *Pst*I and *Nae*I and also by PCR. Chromosomes were counted in four selected ES clones with one disrupted *PU.1* allele. Three clones (R1.9, R9.1, R10.11) containing 40 chromosomes were injected into C57Bl/6 blastocysts. These gave rise to 12 chimeric offspring which were bred with C57Bl/6 mice. F1 mice with germline transmission of the disrupted *PU.1* allele were then bred to produce F2 mice homozygous for the disrupted *PU.1* allele. Mice were genotyped by PCR.

Enzyme histochemistry

Acetone–formaldehyde-fixed smears and formalin-fixed frozen tissue sections were stained for chloroacetate (specific) esterase using a commercially available kit (Sigma, St Louis, MO). Similarly fixed smears were stained for non-specific esterase also using a kit (Sigma, St Louis, MO), utilizing α-naphthyl butyrate as substrate to maximize monocyte/macrophage specificity (Yam *et al.*, 1971). Slides were counterstained with Gill's hematoxylin.

Immunohistochemistry

Slides were stained using the avidin–biotin–peroxidase method (Hsu *et al.*, 1981). Sections were deparaffinized and washed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched by 30 min incubation in 0.3% H_2O_2 in methanol. Slides were blocked with 10% normal rabbit serum for 2 h. F4/80 (clone C1:A3-1, Biosource International, Camarillo, CA) was used at 1:50 dilution; slides were incubated overnight in a humid chamber at 4°C. Secondary antibody and avidin–biotin–peroxidase reagent were obtained commercially (Vector Labs, Burlingame, CA) and used as directed. Diaminobenzidine tetrahydrochloride (DAB) was used for signal detection (Vector labs). Slides were counterstained with Gill's hematoxylin for 30 s.

Flow cytometry

Cell surface fluorescence was determined using fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled antibodies directed against B220 (6B2), BP1 (6C3), CD18, CD43 (S7), HSA (M1/69), μ (33.60), Mac-1 (M1/70), Gr-1 (RB6–8C5) and TCR α/β (H57–597) (PharMingen, San Diego, CA) or CD4 (GK 1.5) and CD8 (53–6.7) (Becton-Dickinson, Mountain View, CA) with the appropriate isotype controls. The combination of anti-B220 and anti-CD43 was achieved using biotin-conjugated 6B2 and streptavidin–FITC along with PE-conjugated S7. Flow cytometry was carried out at The Scripps Research Institute and the Wellesley Hospital Research Institute. For flow cytometry samples, erythrocytes were first removed by lysis or by density separation using Lympholyte M (Cedar Lane Labs, Ontario, Canada), cells were then stained with the appropriate monoclonal antibody. From 10 000 to 30 000 events were collected from samples using a Becton-Dickinson FACScan (Becton-Dickinson), and analysis was performed using Cell Quest (Becton-Dickinson).

Acknowledgements

We gratefully acknowledge Kathy Soderberg for her advice and assistance with the ES cell culture, Jackie Avis for blastocyst injection and Rhonda Jenkins and Mark Herrick for animal care. We also acknowledge the expert technical assistance of Michelle Butler, Kris Conners, Caren Furlonger, Kent Smith and John Knight. We would like to thank Drs David Hume, Dan Tenen and Donald Mosier for helpful discussions and Drs Donald Mosier and Barbara Kee for critically reading the manuscript. This work was supported by NIH Grants DK49886 (B.E.T.) and AI30656 (R.A.M.) and grants from the Medical Research Council of Canada and the National Institute of Canada (C.J.P. and G.E.W.).

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Received on February 13, 1996; revised on June 28, 1996