PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis

Aleksandar Dakic, Donald Metcalf, Ladina Di Rago, Sandra Mifsud, Li Wu, and Stephen L. Nutt

The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia

Although the transcription factor PU.1 is essential for fetal lymphomyelopoiesis, we unexpectedly found that elimination of the gene in adult mice allowed disturbed hematopoiesis, dominated by granulocyte production. Impaired production of lymphocytes was evident in PU.1-deficient bone marrow (BM), but myelocytes and clonogenic granulocytic progenitors that are responsive to granulocyte colony-stimulating factor or interleukin-3 increased dramatically. No identifiable common lymphoid or myeloid progenitor populations were discernable by flow cytometry; however, clonogenic assays suggested an overall increased frequency of blast colony-forming cells and BM chimeras revealed existence of long-term self-renewing PU.1-deficient cells that required PU.1 for lymphoid, but not granulocyte, generation. PU.1 deletion in granulocyte-macrophage progenitors, but not in common myeloid progenitors, resulted in excess granulocyte production; this suggested specific roles of PU.1 at different stages of myeloid development. These findings emphasize the distinct nature of adult hematopoiesis and reveal that PU.1 regulates the specification of the multipotent lymphoid and myeloid compartments and restrains, rather than promotes, granulopoiesis.

CORRESPONDENCE
Stephen L. Nutt:
nutt@wehi.edu.au

Abbreviations used: AML, acute myeloid leukemia; C/EBPα, CCAAT/enhancer binding protein α; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; EPO, erythropoietin; ES, embryonic stem; G-CSF, granulocyte colony-stimulating factor; GMP, granulocyte-macrophage progenitor; HSC, hematopoietic stem cell; IRES, internal ribosome entry site; IRF, interferon regulatory factor; lin⁻, lineage-negative; LF, lactoferrin; LIF, leukemia inhibitory factor; M-CSF, macrophage-CSF; MEP, megakaryocyte-erythrocyte progenitor; polyIC, polyinosine-polycytosine; SCF, stem cell factor.

The multipotent hematopoietic stem cell (HSC) undergoes progressive differentiation to produce all of the mature blood lineages. Hematopoiesis is continuous throughout life, dynamic in its response to stress, and is located in multiple anatomic sites. The current model of adult hematopoiesis proposes that the HSC generates the common myeloid progenitor (CMP) that is able to differentiate into the erythromyeloid lineages, and the common lymphoid progenitor (CLP) that produces B, T, natural killer, and dendritic cells (for review see reference 1). The CMP is postulated to form either of two more restricted cell types—the granulocyte-macrophage progenitor (GMP) or the megakaryocyte-erythrocyte progenitor (MEP; reference 2). An important feature of this model is that committed progenitors represent transit amplifying stages and are incapable of self-renewal (2, 3). Hematopoiesis in the fetal liver is less well-characterized, but also is known to include distinct progenitor types, such as bipotent B cell/macrophage and T cell/macrophage progenitors (4–6). In support of these ontogenic differences, several transcription factors that are known to be essential for embryonic hematopoiesis, including acute myeloid leukemia (AML)-1 (7) and SCL (8), display very different phenotypes when deleted in adult mice. Hence, the extrapolation of data on fetal hematopoiesis to the adult is problematic.

PU.1 is an extensively studied hematopoietic-specific Ets family transcription factor that is essential for embryonic lymphoid and myeloid development (for review see reference 9). Within adult hematopoietic progenitors, *PU.1* is expressed in the HSCs and the earliest myeloid and lymphoid progenitors before being down-regulated in MEPs. Upon lineage commitment, *PU.1* is expressed highly by myeloid cells, at low levels in B cells, and silenced in the erythroid and T cell lineages (10). Recent data has suggested that PU.1 is an important tumor suppressor in murine, and possibly human, AML (11, 12). PU.1 is believed to play these important roles by regulating numerous genes within the myeloid and lymphoid lineages, as well as by interacting with several

L. Wu and S.L. Nutt contributed equally to this work.

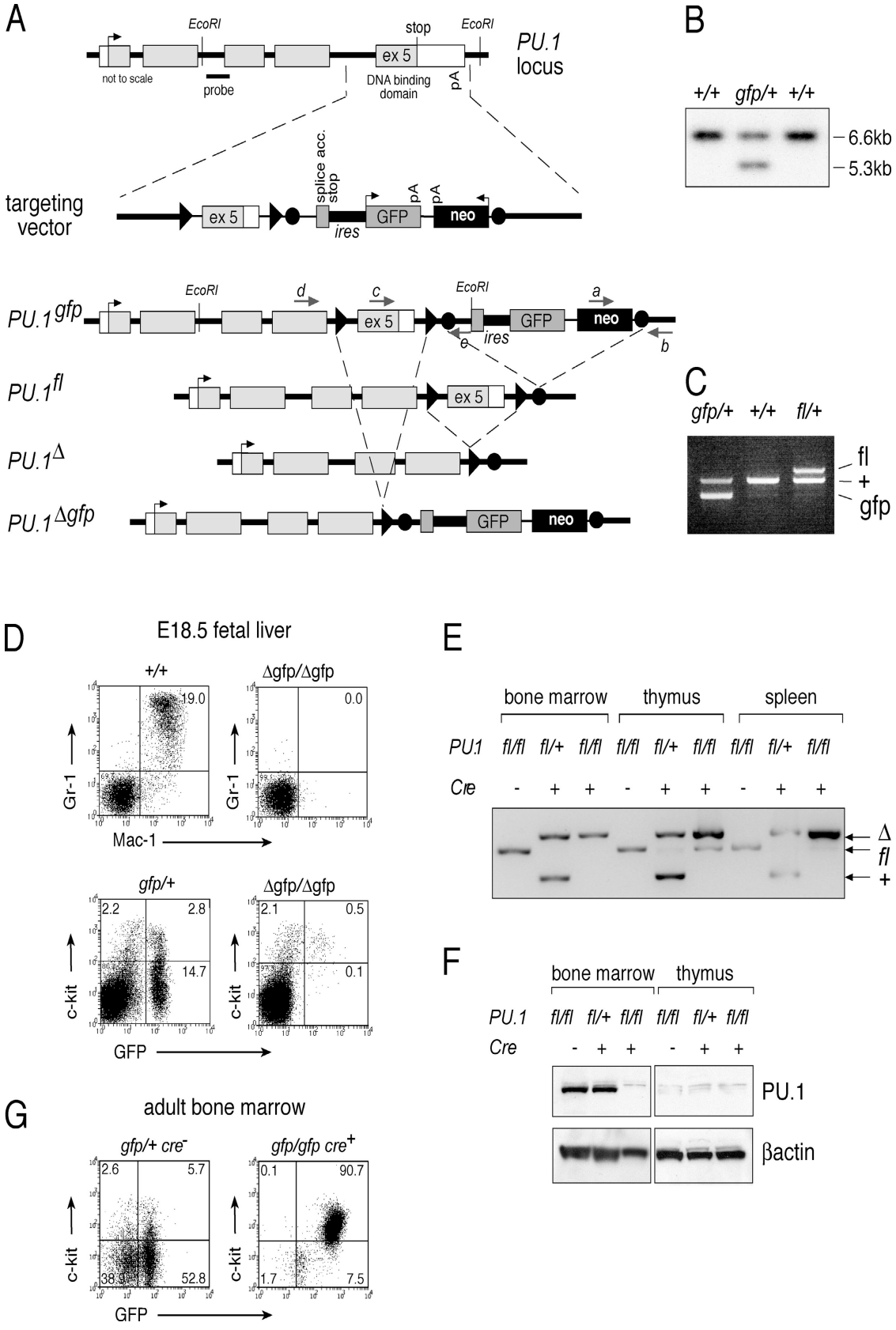

Figure 1. Conditional deletion of *PU.1* in adult hematopoiesis. (A) The genomic locus of *PU.1*. Exons are represented as boxes; introns are represented as black lines; coding regions are in gray; nontranslated regions are in white; arrows indicate the direction of translation. The alleles

other key transcription factors, such as interferon regulatory factor (IRF)4, IRF8, AML-1, CCAAT/enhancer binding protein α (C/EBPα), GATA-1, and c-Jun (13). Because reported PU.1 target genes include *M-CSFR, granulocyte colony-stimulating factor* (G-*CSF)R, GM-CSFRα* (14), and *IL7Rα* (15), cytokine responsiveness is coupled to the action of this developmentally essential transcription factor.

Mice that bear two different *PU.1* mutations have been described extensively. Both lacked granulocytes and macrophages (16, 17), but important differences were reported. The mutant of Scott et al., which died in late gestation (between E17-18), lacked lymphocytes and long-term BM repopulating activity (16, 18), whereas the mutant of McKercher et al. was born alive and could live up to 2 wk on antibiotics (17). Analysis of these pups revealed aberrant B cell development; an abnormally large population of Mac-1$^+$ immature myeloid cells; and delayed, but normal, T lymphopoiesis. Recently, a third mutant was reported that died at 1 d and had defects in erythroid progenitor self-renewal; however, no data on its lymphoid and myeloid phenotypes were reported (19). These studies demonstrate that PU.1 is essential for normal fetal hematopoiesis, but do not address its roles in adult hematopoiesis.

We developed a conditional mutation that allows inactivation of *PU.1* in adult hematopoietic cells. We find that *PU.1* ablation resulted in dramatically perturbed hematopoiesis and, contrary to expectation, greatly enhanced granulopoiesis. These changes were accompanied by a marked expansion in granulocytic progenitors, and loss of macrophage-CSF (M-CSF), GM-CSF and IL-6 responsiveness, whereas blast colony-forming capacity was preserved. Analysis of the multipotent progenitors revealed that PU.1 deficiency resulted in the loss of all FACS-identifiable lymphoid and myeloid progenitor populations. These results suggest that PU.1 is essential for normal transit through the CLP and CMP/GMP stages of adult hematopoiesis where it promotes lymphopoiesis, and conversely, restricts granulopoiesis.

RESULTS

Generation and validation of a PU.1 conditional allele

The PU.1-targeting construct contained *LoxP* flanked *PU.1* exon-5 followed by an *frt* flanked *internal ribosome entry site (IRES)-GFP* cassette (Fig. 1 A). Homologous recombination in embryonic stem (ES) cells was confirmed using Southern hybridization and PCR genotyping, producing the *PU.1^{gfp}* allele (Fig. 1, A–C). Germ-line transmission was achieved with two independent ES cell clones with identical GFP expression and null phenotype. The GFP reporter allowed detailed characterization of the *PU.1* expression domain, which was reported elsewhere (10). The conditional *PU.1^{fl}* allele was produced by breeding *PU.1^{gfp}* with a Flp recombinase-expressing strain (Fig. 1 A).

To determine the phenotype of this *PU.1* mutation, we bred the *PU.1^{gfp}* or *PU.1^{fl}* allele to the Deleter-Cre strain to produce *PU.1$^{\Delta gfp}$* or *PU.1$^{\Delta}$*, respectively. Intercrossing of *PU.1$^{\Delta/+}$* mice resulted in no *PU.1$^{\Delta/\Delta}$* individuals at day 21, whereas E18.5 *PU.1$^{\Delta/\Delta}$* embryos were present at the expected frequency (+/+ = 13 [24%], +/Δ = 30 [57%], Δ/Δ = 10 [19%]). Timed pregnancies were allowed to continue to term, but *PU.1$^{\Delta/\Delta}$* pups died shortly after birth (unpublished data). Analysis of *PU.1$^{\Delta/\Delta}$* E18.5 embryos revealed an absence of myeloid (Mac-1$^+$Gr-1$^+$) lineage cells (Fig. 1 D). Although *PU.1$^{\Delta gfp/+}$* embryos had populations of GFP^{high} myeloid and GFP^{low} B cells, as well as GFP$^+$c-kit$^+$ progenitors, *PU.1$^{\Delta gfp/\Delta gfp}$* embryos showed only a residual GFP$^+$c-kit$^+$ population (Fig. 1 D). This null phenotype was most similar to that of Scott et al. (16) and showed that PU.1 was required for the existence of virtually almost all *PU.1*-expressing cells in the fetal liver.

PU.1 inactivation perturbs adult hematopoiesis

To delete *PU.1* conditionally in adult hematopoietic cells, *PU.1$^{fl/fl}$* mice were crossed to the IFNαβ-inducible *MxCre* transgene, which can be activated efficiently by polyinosine-polycytosine (polyIC; reference 20). *PU.1$^{fl/fl}$* MxCre$^+$ and *MxCre$^-$* mice were injected i.p. with polyIC at days 0 and 3 and analyzed 10–14 d after induction. Exon-5 deletion frequency was monitored using a PCR assay on genomic DNA and confirmed by Western blotting (Fig. 1, E and F). Typically, at day 10 after injection, the deletion frequency approached 100% in BM and was between 70–90% in spleen, blood, and thymus (Fig. 1 E). In contrast to the loss of fetal *PU.1*-expressing cells, *PU.1*-deficient adult BM contained a population of uniformly GFP^{high} cells; this suggested that PU.1 was not required for the survival of all adult hematopoietic cells (Fig. 1 G).

Analysis of *PU.1$^{\Delta/\Delta}$* mice at 2 wk after polyIC treatment revealed several hematologic abnormalities. The BM contained normal total cellularity but exhibited an increased proportion of blast cells, and immature cells of the granulocyte

derived from the integration of the targeting vector and subsequent manipulations are shown. pA, polyadenylation signal sequence; circles, *frt* sites; triangles, *loxP* sites; stop, stop codons in all reading frames; splice acc., splice acceptor. Targeted *PU.1^{gfp}* alleles can be converted by flp-mediated deletion to *PU.1^{fl}* or Cre deletion to *PU.1$^{\Delta}$*. The position and direction of the genotyping primers (*a–e*) and *Eco RI* sites are indicated. (B) Southern blot analysis of ES cell *Eco RI*-digested DNA showing the wild-type (6.6 kb) and targeted (5.3 kb) alleles (C) PCR genotyping of tail DNA using the primer set *a/b/c* showing the correct amplification of the wild-type, *PU.1^{gfp}*, and *PU.1^{fl}* alleles. (D) Flow cytometric analysis of E18.5 fetal liver from *PU.1$^{\Delta gfp/+}$* intercrosses showing loss of myeloid cells in the absence of PU.1 and the GFP fluorescence in progenitors (c-kit$^+$). (E) PCR showing the relative polyIC-inducible deletion of the *PU.1^{fl}* allele in vivo. Mice were injected with polyIC on days 0 and 3 and analyzed on day 10. DNA was amplified with the primer combination *b/c/d*. (F) Western blot analysis of PU.1 of mice of the indicated genotypes. Equal loading was confirmed using β-actin. PU.1 is not expressed in most thymocytes and demonstrates nonspecific antibody binding. (G) GFP fluorescence in adult BM. *PU.1$^{\Delta gfp/\Delta gfp}$* (*gfp/gfp MxCre$^+$*) and control (*gfp/+ MxCre$^-$*) mice are shown at 2 wk after polyIC injection.

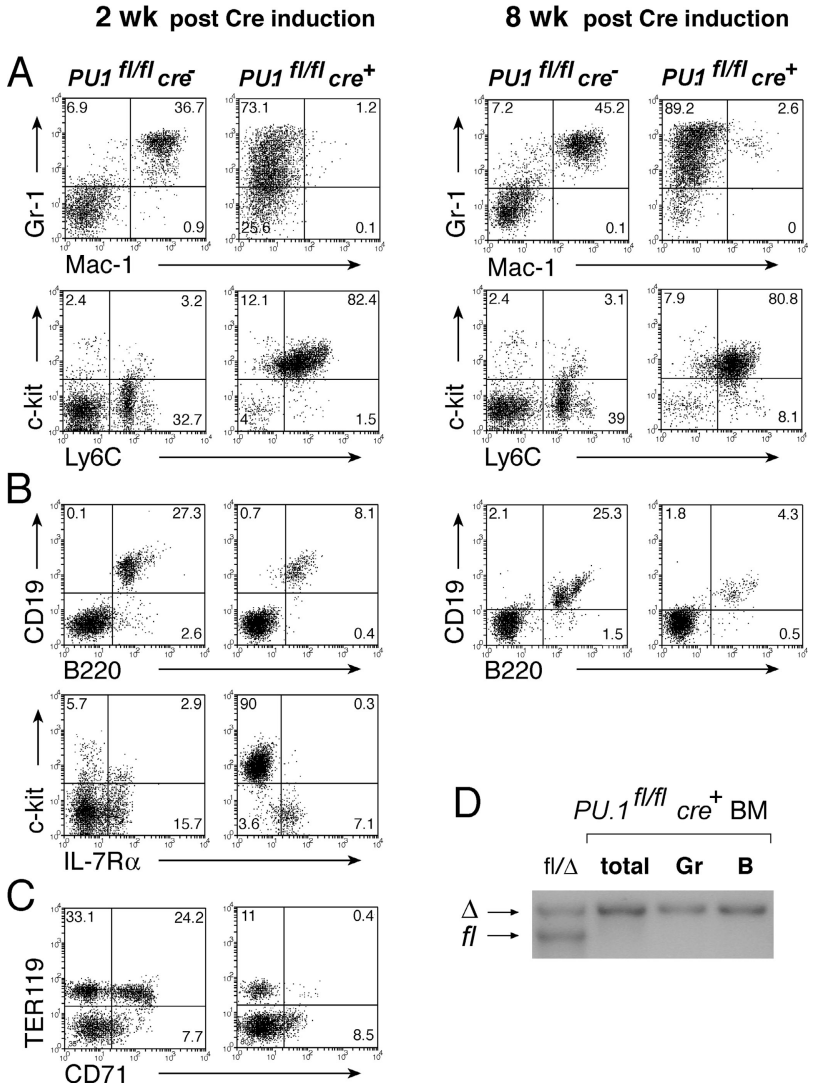

Figure 2. PU.1 inactivation results in relative expansion of granulopoiesis. Representative analysis of the (A) myeloid, (B) B lymphoid, and (C) erythroid lineages from the BM of *PU.1*$^{\Delta/\Delta}$ (*fl/fl MxCre*$^{+}$) and control (*fl/fl MxCre*$^{-}$) mice. Mice were injected on days 0 and 3 with polyIC and analyzed after 2 or 8 wk. (D) PCR analysis showing the relative PU.1 deletion of total BM and sorted cells of the indicated lineages 2 wk after polyIC treatment. B cells were identified as CD19^{+}B220^{+}, granulocytes as Gr1^{+}. *PU.1*$^{fl/\Delta}$ tail DNA was used as a control.

lineage (promyelocytes and myelocytes; Table I). Staining with myeloid lineage markers showed that the mutant mice had twice as many Gr-1^{+} cells as controls and were Mac-1^{-} (Fig. 2 A). Genotype analysis of total BM, granulocytes, and B cells revealed virtually complete gene deletion at this time point (Fig. 2 D). Strikingly, almost all BM cells of *PU.1*$^{\Delta/\Delta}$ mice expressed Ly6C and c-kit, markers that are indicative of myeloid precursors. This expansion of PU.1-deleted (Mac-1^{-}) granulocytic precursors in the BM also was apparent 8 wk after the last polyIC injection. At this time point, some differentiation of PU.1 wild-type cells can be seen by the reappearance of a small number of Mac-1^{+} cells (Fig. 2 A). Although Mac-1, F4/80, and FcγRII/III staining was lost from *PU.1*-deleted hematopoietic organs (Fig. 2 A) which indicate efficient gene deletion, morphologically recognizable monocytes remained (Table I). In contrast to the expanded granulocytic lineage population, B lymphocyte numbers were reduced variably in the *PU.1*$^{\Delta/\Delta}$ BM at day 14, with most cells having a preB cell phenotype (CD19^{+}B220^{+}IL7Rα^{+}c-kit^{-}) and displaying complete deletion of exon-5 (Fig. 2, B and D). PolyIC induces an IFN response that is particularly detrimental to BM B cell precursors (21). Therefore, the differences at this early time point may represent differential repopulation of the B lineage (after the IFN response) and a consequence of excessive granulopoiesis in the BM. *PU.1*$^{\Delta/\Delta}$ BM also lacked the nucleated erythroid cells that represented 17% of the control BM at 2 weeks (Table I). FACS analysis confirmed the *PU.1*$^{\Delta/\Delta}$ BM to be markedly deficient in Ter119^{+}CD71^{+} erythroblasts (Fig. 2 C). The loss of nucleated erythroid cells also was evident in histologic sections of the mutant BM (Fig.

Table I. Cellular analysis of hematological organs

Parameter	2 wk after induction		8 wk after induction	
	PU.1$^{fl/fl}$ MxCre^{+}	PU.1$^{fl/fl}$ MxCre^{-}	PU.1$^{fl/fl}$ MxCre^{+}	PU.1$^{fl/fl}$ MxCre^{-}
	n = 5	*n* = 5	*n* = 3	*n* = 3
Peripheral blood				
Total cells	3,230 ± 1,660	4,350 ± 2,340	4,343 ± 2,759	8,420 ± 5,274
Neutrophils	160 ± 280	850 ± 370	36 ± 35	407 ± 230
Lymphocytes	2,910 ± 1,330	2,870 ± 1,340	3,403 ± 1,442	7,513 ± 4,702
Monocytes	100 ± 70	590 ± 750	237 ± 251	310 ± 185
Eosinophils	50 ± 100	30 ± 50	0 ± 0	183 ± 275
Platelets $\times 10^{-5}$	6.3 ± 1.0	10.7 ± 3.3	13.7 ± 2.1	9.5 ± 2.1
Hematocrit %	44 ± 3	43 ± 3	43 ± 3.6	46 ± 1.2
Spleen				
Weight mg	149 ± 46	110 ± 18	138 ± 2.2	95.3 ± 15
Blast cells	5 ± 2	3 ± 1	2 ± 0	4 ± 0.6
Myelocytes	3 ± 2	1 ± 1	1 ± 1	0
Neutrophils	3 ± 1	5 ± 4	5 ± 2	1 ± 1
Lymphocytes	59 ± 16	59 ± 14	60 ± 16	89 ± 0.6
Monocytes	2 ± 2	4 ± 3	1 ± 0	2 ± 2
Eosinophils	1 ± 1	1 ± 1	0.3 ± 0.6	0.3 ± 0.6
Nucleated erythroid	26 ± 12	27 ± 12	31 ± 13	3.7 ± 2.3
Bone marrow				
Total cells $\times 10^{-6}$	44.4 ± 8.5	42.3 ± 6.0	37.9 ± 5.6	33.4 ± 1.5
Blast cells	9 ± 5	3 ± 0	7 ± 1	4.3 ± 1.2
Myelocytes	25 ± 4	9 ± 5	14 ± 0.6	10 ± 3.6
Neutrophils	45 ± 5	48 ± 12	49 ± 16	35 ± 4.4
Lymphocytes	14 ± 6	12 ± 10	19 ± 10	23 ± 2.1
Monocytes	7 ± 5	8 ± 4	5.7 ± 2.5	9.7 ± 1.2
Eosinophils	0 ± 0	3 ± 2	0.3 ± 0.6	2 ± 1
Nucleated erythroid	0.4 ± 0.6	17 ± 9	5.7 ± 9.8	16 ± 5.5

Data are the mean values ± SD. *n* = 5 mice for 2-wk and *n* = 3 mice for 8-wk postinduction experiments. Peripheral blood values are per microliter. Total BM cells are per femur. Cellular differentials for BM and spleen are shown as percentages. Myelocytes include promyelocytes and myelocytes. Neutrophils include metamyelocytes, band neutrophils, and neutrophils.

3 A) and similarly apparent after 8 wk (Table I). Conversely, nucleated erythroid cells were present in spleens of both genotypes at 2 wk, but remained only in the *PU.1*$^{\Delta/\Delta}$ spleens at 8 wk after treatment (Table I). The presence of nucleated erythrocytes in spleen at the early time point most likely reflected the response to polyIC treatment, whereas their long-term presence in the case of *PU.1*$^{\Delta/\Delta}$ mice represented true extramedullary hematopoiesis.

The spleen of *PU.1*$^{\Delta/\Delta}$ mice also contained increased numbers of granulocytic cells, although their frequency was variable (20–90% Gr-1^{+}c-kit^{+}; Fig. 3, B and C). In more severe cases (Fig. 3, B and C), spleen histology showed homogeneous infiltration by immature granulocytic forms and loss of splenic architecture. PCR analysis confirmed complete deletion of exon-5 in splenic granulocytes (Fig. 3 C).

Absolute spleen lymphocyte numbers were relatively normal in most mice with a two- to threefold percentage decrease in individuals with the most pronounced granulocyte expansion (Table I and Fig. 3, A–C). PU.1 deletion in the splenic lymphocytes was less frequent (50–70%), although sorting of *PU.1*$^{\Delta/\Delta_{gfp}}$ mature B cells based on GFP expression (that increases upon exon-5 deletion [Fig. 1 G]) showed that all GFP^{high} cells were PU.1 deficient (Fig. 3 C).

Granulocyte maturation in the absence of PU.1

The expanded number of morphologically identifiable granulocytes suggested that in contrast with fetal development, PU.1 is not required for the differentiation of this lineage in vivo. To examine the maturation of *PU.1*-deficient granulocytes, Gr-1^{+} BM cells were cultured for 7 d in G-CSF before being subjected to flow cytometry, cytospin, and molecular analysis. In agreement with the loss of Mac-1^{+} staining, *PU.1*$^{\Delta/\Delta}$ granulocytes represented a pure population of deleted cells (Fig. 3 D). *PU.1*$^{\Delta/\Delta}$ granulocytes, in contrast with wild-type controls, maintained c-kit expression and displayed some, but not complete, maturation as measured by morphologic criteria (Fig. 3

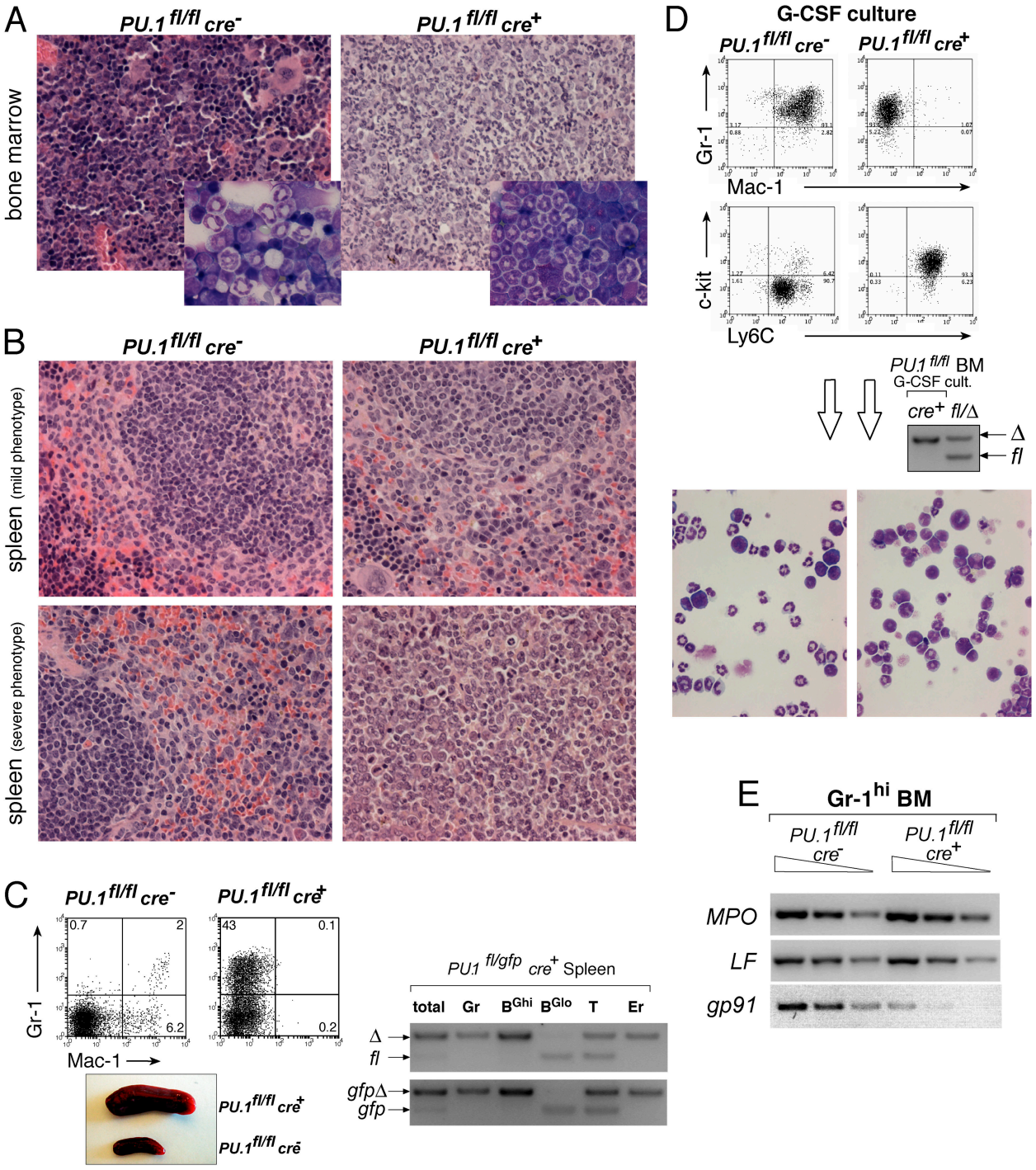

Figure 3. Perturbed granulocytic differentiation in the absence of PU.1. (A) Histologic section of day 21 BM from mice of the indicated genotype. Insets show cytocentrifuge preparations of corresponding BM. (B) Histologic section of relatively mild and severe granulocytic infiltrations in the spleen at day 21. (C) Severe *PU.1*$^{\Delta/\Delta}$ (*fl/fl MxCre*$^{+}$) mice display splenomegaly and enhanced percentage of Gr-1^{+}Mac-1^{-} cells by flow cytometry. PCR showing efficient deletion of *PU.1*^{fl} and PU.1^{gfp} alleles in 2-wk spleen of a *PU.1*$^{\Delta/\Delta gfp}$ (*fl/gfp MxCre*$^{+}$) mouse. Total, total splenocytes; Gr, Gr-1^{+} cells; B, CD19^{+}B220^{+} B cells; T, CD3^{+} T cells; Er, CD71^{+}Ter119^{+} nucleated erythrocytes. B cells also were gated on GFP^{high} (B^{Ghi}) or GFP^{low} (B^{Glo}). The *fl* and *gfp* alleles were amplified with the primer combination *b/c/d* and *e/c/d*, respectively. (D) Control (*fl/fl MxCre*$^{-}$) and *PU.1*$^{\Delta/\Delta}$ (*fl/fl MxCre*$^{+}$) granulocytes were grown in G-CSF for 7 d before being analyzed by flow cytometry and morphology. PCR analysis showed the complete *PU.1* deletion of cultured granulocytes. (E) BM Gr-1^{hi} cells were sorted and analyzed by RT-PCR for the expression of granulocytic enzymes, myeloperoxidase (MPO), LF, and gp91.

Table II. In vitro analysis of bone marrow colony-forming progenitors

Genotype ($n = 4$)	Stimulus	Mean no. of colonies					
		Blast	G	GM	M	Eo	Meg
PU.1$^{fl/fl}$ *MxCre*$^{+}$	GM-CSF		2 ± 2	0.3 ± 0.5	4 ± 6	0 ± 0	
	G-CSF		**83 ± 31**	0 ± 0	0 ± 0		
	M-CSF		0.5 ± 1.0	0 ± 0	1.5 ± 2.4		
	IL-3	12 ± 12	**152 ± 15**	2 ± 1	6 ± 5	0 ± 0	0.5 ± 1.0
	SCF	5 ± 5	29 ± 8	0 ± 0	0 ± 0		
	IL-6		0.8 ± 0.7	0 ± 0	0 ± 0		
	SCF + G-CSF	3 ± 4	**129 ± 15**	0 ± 0	0 ± 0		
	FL + LIF	0 ± 0	0.2 ± 0.2	0 ± 0	0 ± 0		
	IFNγ		14 ± 11	0 ± 0	0 ± 0		
	EPO						0.1 ± 0.1
	TPO						1 ± 1
	SCF + IL-3 + EPO	6 ± 9	**178 ± 51**	7 ± 5	4 ± 4	0 ± 0	2 ± 1
	saline		0.8 ± 1.0	0 ± 0	0 ± 0		
PU.1$^{fl/fl}$ *MxCre*$^{-}$	GM-CSF		19 ± 5	4 ± 4	31 ± 13	3 ± 2	
	G-CSF		**10 ± 3**	0 ± 0	2 ± 3		
	M-CSF		1 ± 1	5 ± 3	69 ± 29		
	IL-3	8 ± 7	**18 ± 6**	11 ± 5	27 ± 9	2 ± 1	4 ± 3
	SCF	5 ± 3	14 ± 7	1 ± 1	1 ± 1		
	IL-6		8 ± 4	1 ± 1	0.5 ± 0.6		
	SCF + G-CSF	7 ± 3	**18 ± 11**	3 ± 2	3 ± 3		
	FL + LIF	2 ± 1	0.8 ± 0.5	0 ± 0	0.1 ± 0.3		
	IFNγ		3 ± 2	0 ± 0	0 ± 0		
	EPO						5 ± 2
	TPO						2 ± 1
	SCF + IL-3 + EPO	7 ± 3	**21 ± 8**	11 ± 6	24 ± 9	2 ± 1	16 ± 6
	saline		0 ± 0	0 ± 0	0 ± 0		

Bone marrow of *PU.1*$^{\Delta/\Delta}$ (*fl/fl MxCre*$^{+}$) and control (*fl/fl MxCre*$^{-}$) mice was analyzed on day 14 after induction. 25,000 cells were cultured in agar with the indicated cytokines for 7 d, stained, and counted. Experiments were performed using four separate mice and quadruplicate cultures for each stimulus. Numbers represent mean colony numbers from four mice ± SD. Values in bold highlight examples of increased numbers of committed colony-forming progenitors responsive to selected growth factors or growth factor combinations after *PU.1* deletion.

Eo, eosinophil colony; FL, FH3 ligand; G, granulocyte colony; GM, granulocyte/macrophage colony; M, macrophage colony; Meg, megakaryocyte colony; TPO, thrombopoietin.

D). RT-PCR analysis of the sorted Gr-1^{hi} cells revealed that the primary granule component, *myeloperoxidase*, as well as the secondary granule component, *lactoferrin* (*LF*), were expressed normally, whereas the putative PU.1 target gene and component of nicotinamide adenine dinucleotide phosphate oxidase, *gp91* (22), was down-regulated (Fig. 3 E). The normal expression of *LF* contrasted with the absence of this transcript in *PU.1*$^{-/-}$ fetal liver-derived cultures (30). These data suggest that *PU.1*$^{\Delta/\Delta}$ granulocytes were capable of aberrant, and possibly delayed, differentiation to relatively mature cells (Fig. 3, A inset, D, E), despite the reported requirement for PU.1 in the transcription of a variety of genes that is required for granulocyte function.

Expanded clonogenic granulocyte progenitors in the absence of PU.1

Progenitor cells in the BM and spleen of *PU.1*$^{\Delta/\Delta}$ mice were analyzed at 2 and 8 wk after PU.1 ablation, using agar cultures and a range of stimulating factors. At the earlier time point, the mutant BM was grossly depleted in cells that were able to respond to GM-CSF or M-CSF by forming colonies or clusters (Table II). In sharp contrast, *PU.1*$^{\Delta/\Delta}$ cultures that were stimulated by G-CSF developed greatly elevated numbers of granulocytic colonies, the cells of which showed some morphologic maturation (Fig. 3 D). Excessive numbers of granulocytic colonies also developed in cultures that were stimulated by IL-3 or stem cell factor (SCF; c-kit L), although a few macrophage-containing colonies did develop in IL-3–stimulated cultures. In contrast with the excessive granulocyte colony numbers that developed with G-CSF, virtually no colonies arose in response to IL-6. Moreover, the *PU.1*$^{\Delta/\Delta}$ BM lacked eosinophil colony-forming cells that were responsive to GM-CSF, IL-3, or IL-5 (Table II). In cultures that were stimulated by the optimal growth factor combination for megakaryocyte colony formation (SCF +

Table III. In vitro analysis of spleen colony-forming progenitors

Genotype ($n = 4$)	Stimulus	Mean no. of colonies					
		Blast	G	GM	M	Eo	Meg
PU.1$^{fl/fl}$ *MxCre*$^{+}$	G-CSF		**118 ± 96**	2 ± 2	1 ± 1		
	IL-3	26 ± 26	**269 ± 200**	27 ± 18	**59 ± 38**	0 ± 0	19 ± 14
	SCF + IL-3 + EPO	16 ± 21	**247 ± 154**	28 ± 20	**60 ± 47**	0 ± 0	74 ± 52
PU.1$^{fl/fl}$ *MxCre*$^{-}$	G-CSF		**3 ± 5**	0 ± 0	0 ± 0		
	IL-3	1 ± 2	**7 ± 11**	4 ± 5	**10 ± 11**	0.5 ± 1.0	0.8 ± 1.0
	SCF + IL-3 + EPO	2 ± 2	**11 ± 11**	5 ± 5	**6 ± 6**	0.5 ± 0.6	11 ± 9

Mice were treated identically to those in Table II. Each culture contained 50,000 spleen cells. Numbers represent the mean ± SD of colony counts from four mice of each genotype. Values in bold highlight examples of increased numbers of committed colony-forming progenitors responsive to selected growth factors or growth factor combinations after *PU.1* deletion.

Eo, eosinophil colony; G, granulocyte colony; GM, granulocyte/macrophage colony; M, macrophage colony; Meg, megakaryocyte colony.

IL-3 + erythropoietin [EPO]), few or no megakaryocyte colonies developed in cultures of *PU.1*$^{\Delta/\Delta}$ BM cells. PCR analysis of BM (Fig. 2 D) and individual colonies ($n = 81$) showed the nearly total deletion of exon-5; this confirmed that any colony formation was the result of PU.1-deficient progenitors (unpublished data).

Analysis of clonogenic BM progenitors 8 wk after polyIC treatment showed a similar trend toward excess granulocytic progenitor numbers. However, G-CSF–responsive progenitors were present in only slightly higher numbers (*fl/fl* 8.6 ± 3.8, *Δ/Δ* 16 ± 4.7), whereas IL-3 (*fl/fl* 15 ± 10, *Δ/Δ* 95 ± 22), SCF/G-CSF (*fl/fl* 20 ± 5.0, *Δ/Δ* 48 ± 13), and SCF/IL-3/EPO (*fl/fl* 16 ± 3.6, *Δ/Δ* 97 ± 8.9) cultures generated increased numbers of granulocytic colonies (mean ± SD; $n = 3$ for each genotype).

At 2 wk after polyIC treatment, the spleen in control mice was enlarged and contained elevated numbers of colony-forming cells. Strikingly, mutant spleen cells generated vastly increased numbers of granulocytic colonies (Table III), excessive numbers of megakaryocytic colonies, and, interestingly, IL-3–responsive macrophage-containing colonies. These data demonstrate that PU.1 deficiency induced extramedullary hematopoiesis that, similarly to BM, resulted in excessive granulopoiesis. The spleen also contained significant numbers of IL-3, but not M-CSF–responsive macrophage colony-forming cells. This was despite the fact that the deletion frequency was high and no Mac-1^{+} cells were present (Fig. 3 C; Table III). This effect largely was transient because many fewer clonogenic cells were found in the spleen at the 8-wk time point. However, increased SCF/IL-3/EPO–responsive granulocyte (*fl/fl* 1 ± 1, *Δ/Δ* 25 ± 7.2) and megakaryocyte (*fl/fl* 3.7 ± 2.9, *Δ/Δ* 12 ± 7.5) colonies were still present in the mutant spleens; this was indicative of ongoing hematopoiesis (mean ± SD; $n = 3$ for each group). Collectively, these data demonstrate that PU.1 inactivation resulted in rapid and dramatic changes in the responsiveness, number, and anatomic location of hematopoietic progenitors.

PU.1 is required for the balanced production of multipotent bone marrow progenitors

To determine directly the effect of *PU.1* deletion on defined BM progenitors, we isolated lineage-negative (lin^{-}) cells 2 wk after Cre activation, a time point when gene deletion is essentially complete (Fig. 4 B). PU.1 deficiency resulted in a dramatic loss of any readily identifiable lymphoid and myeloid progenitor or stem cell populations (Fig. 4 A). Lin^{-} cells were c-kit^{+}/Sca1^{-}/IL-7Rα^{-}/CD34^{-}/FcγRII/III^{low}, a phenotype that is not defined by the existing progenitor scheme (Fig. 4 A). Morphologic analysis of sorted lin^{-} cells revealed a high proportion of blast cells in either genotype with a slightly higher proportion in the S+G_2+M phases of the cell cycle in the *PU.1*$^{\Delta/\Delta}$ preparations (Fig. 4 B). All *PU.1*$^{\Delta/\Delta}$ lin^{-} cells expressed high levels of GFP, which indicated that such cells can survive in the absence of PU.1 (Fig. 4 A) and contrasts with a report that PU.1 positively regulates its own promoter (23).

RT-PCR analysis of lin^{-} BM cells revealed that PU.1-deficient cells had little *M-CSFR*, *GM-CSFRα*, or *IL-5Rα* expression, and also lacked *IL-6Rα* and *gp130*, the common component of the IL-6R and leukemia inhibitory factor (LIF) receptor (Fig. 4 C). It also was of note that *PU.1*$^{\Delta/\Delta}$ cells showed decreased expression of a variety of important transcription factors that have been shown to interact functionally with PU.1, including *GATA-1* and *IRF-8* (Fig. 4 C). The levels of *GATA-1* and *IRF-8* most likely reflected the expansion of the granulocytic lineage and absence of nucleated erythrocytes in BM. The possibility that the Ets family members, Spi-B or Spi-C, compensate for some PU.1 functions was excluded because neither was expressed in *PU.1*$^{\Delta/\Delta}$ lin^{-} BM. Several other critical genes, such as *GM-CSFRβ*, *IL-2Rγ*, *GATA-2*, and *ikaros*, were similarly expressed in cells of both genotypes (Fig. 4 C). Thus, in agreement with the altered in vitro growth characteristics, *PU.1*$^{\Delta/\Delta}$ progenitors displayed specific alterations in their transcriptional profile as compared with wild-type cells.

The apparent loss of FACS-identifiable progenitors simply could be the consequence of the loss of expression of pu-

tative PU.1 target genes, such as *IL-7Rα* (15) and *FcγRIIIa* (24). To overcome this uncertainty and to determine the source of the excess granulocytic cells that were observed, we sorted myeloid progenitors at short time intervals after PU.1 deletion. Mice were injected with polyIC 36 h and 24 h before the isolation of the defined myeloid and erythroid progenitors. This short time frame was sufficient for efficient deletion of exon-5 in CMP and GMP, but not for the loss of the critical cell surface markers (Fig. 5). At this stage, *cre*$^{+}$-sorted CMP and GMP populations were morphologically indistinguishable from their *cre*$^{-}$ counterparts (Fig. 5). Analysis of the in vitro clonogenic potential of sorted CMPs and GMPs confirmed the efficient PU.1 inactivation, because virtually no M-CSF–responsive colonies were obtained from *PU.1*$^{\Delta/\Delta}$ progenitors (Fig. 5). Although the number of granulocyte colonies was relatively similar from *PU.1*$^{fl/fl}$ and *PU.1*$^{\Delta/\Delta}$ CMPs (see G-CSF, IL-3, or IL-3/SCF/EPO), the corresponding cultures of GMPs showed a pronounced enhancement of *PU.1*-deleted granulocyte colonies. As expected, sorted MEP gave rise to very few myeloid colonies in these assays (unpublished data). These data suggest that the enhanced granulopoiesis that was observed in the absence of PU.1 is derived from increased/deregulated differentiation capacity of the GMP, but not the more multipotent CMP.

These experiments gave two additional unexpected findings. First, in contrast with the loss of GM-CSF–responsive

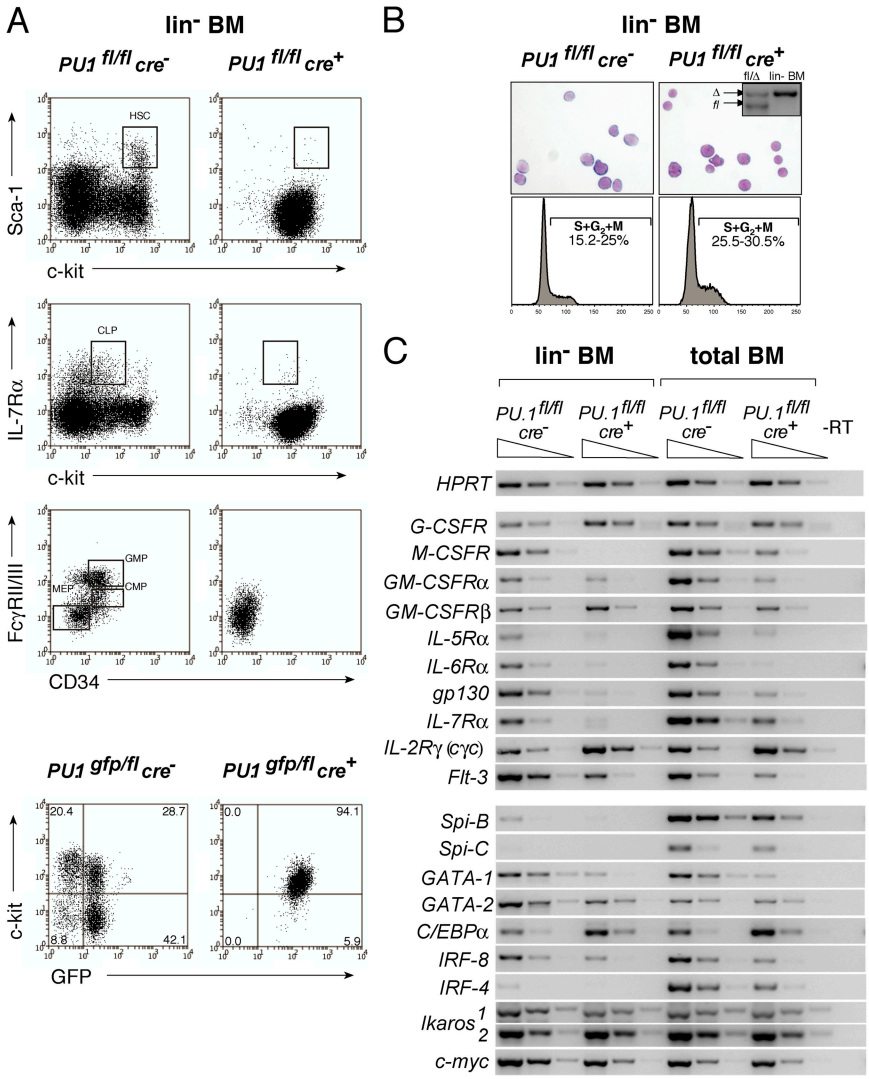

Figure 4. PU.1 is essential for multipotent lymphoid and myeloid progenitors. (A) BM lin^{-} cells from 2-wk *PU.1*$^{\Delta/\Delta}$ (*fl/fl MxCre*$^{+}$) and control (*fl/fl MxCre*$^{-}$) polyIC-treated mice were assayed for HSC and lymphoid and myeloid progenitors. For GFP analysis, *PU.1*$^{gfp/\Delta}$ (*PU.1*$^{gfp/fl}$MxCre^{+}) or control (*PU.1*$^{gfp/fl}$MxCre^{-}) mice were treated as above. (B) Cytocentrifuge preparation and cell cycle analysis of lin^{-} cells. Inset: PCR showing complete deletion of exon-5 in lin^{-} cells. Range of cycling cells (S+G_2+M) observed from two experiments is indicated. (C) RT-PCR for key hematopoietic regulators and PU.1 target genes in lin^{-} and total BM. *HPRT* was used as a control for cDNA input. −RT, no reverse transcriptase was used in the reaction. Samples were taken at four cycle intervals. *Ikaros* isoforms 1 and 2 are indicated.

colonies 2 wk after Cre induction, short-term *PU.1*-deleted GMPs generated excess granulocyte colonies when stimulated with GM-CSF (Fig. 5). A similar trend was seen with total BM cells at this short time point (*fl/fl* 21.2 ± 6.1, *Δ/Δ* 32.9 ± 12; mean ± SD; $n = 3$). We interpret this responsiveness to GM-CSF to indicate that PU.1 may be critical for the expression of the *GM-CSFRα* between HSC and CMP stages (as assayed at the 2-wk time point), but not for the maintenance of its expression in the more mature GMPs (which only can be assayed with short time points before new GM-CSFRα–negative GMPs are generated from HSC/CMP). Second, despite the lack of M-CSF responsiveness, macrophage colonies developed in IL-3 or IL-3/SCF from *PU.1*$^{\Delta/\Delta}$ CMPs, but not GMPs; this suggests that PU.1 loss may have resulted in an earlier than normal switch to granulocyte commitment in this sequence of amplifying myeloid progenitors.

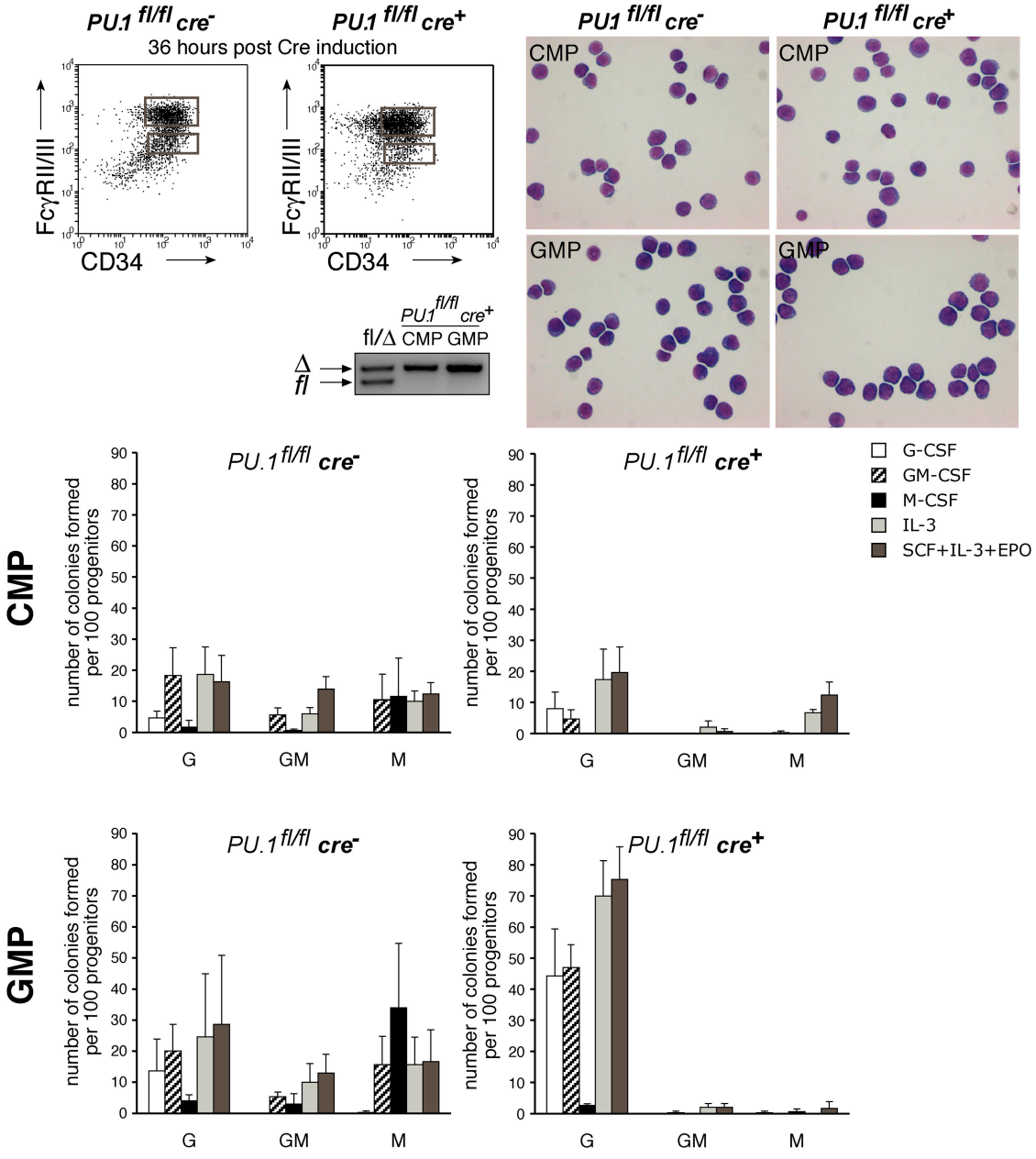

Figure 5. PU.1 regulates granulocyte production from the GMP. BM lin$^-$ cells from 36-h post-polyIC treatment *PU.1*$^{\Delta/\Delta}$ (*fl/fl MxCre*$^+$) and control (*fl/fl MxCre*$^-$) mice were assayed for erythromyeloid progenitors. Sorted CMP and GMP using the indicated gates were subjected to PCR for *PU.1* exon-5 deletion. Cytocentrifuge preparations were stained with May-Grunwald-Giemsa. Sorted cells were cultured in the indicated cytokines for 7 d, stained, and counted. 100 cells were plated in quadruplicate for each stimulus. Numbers represent the mean ± average deviation from three independent experiments.

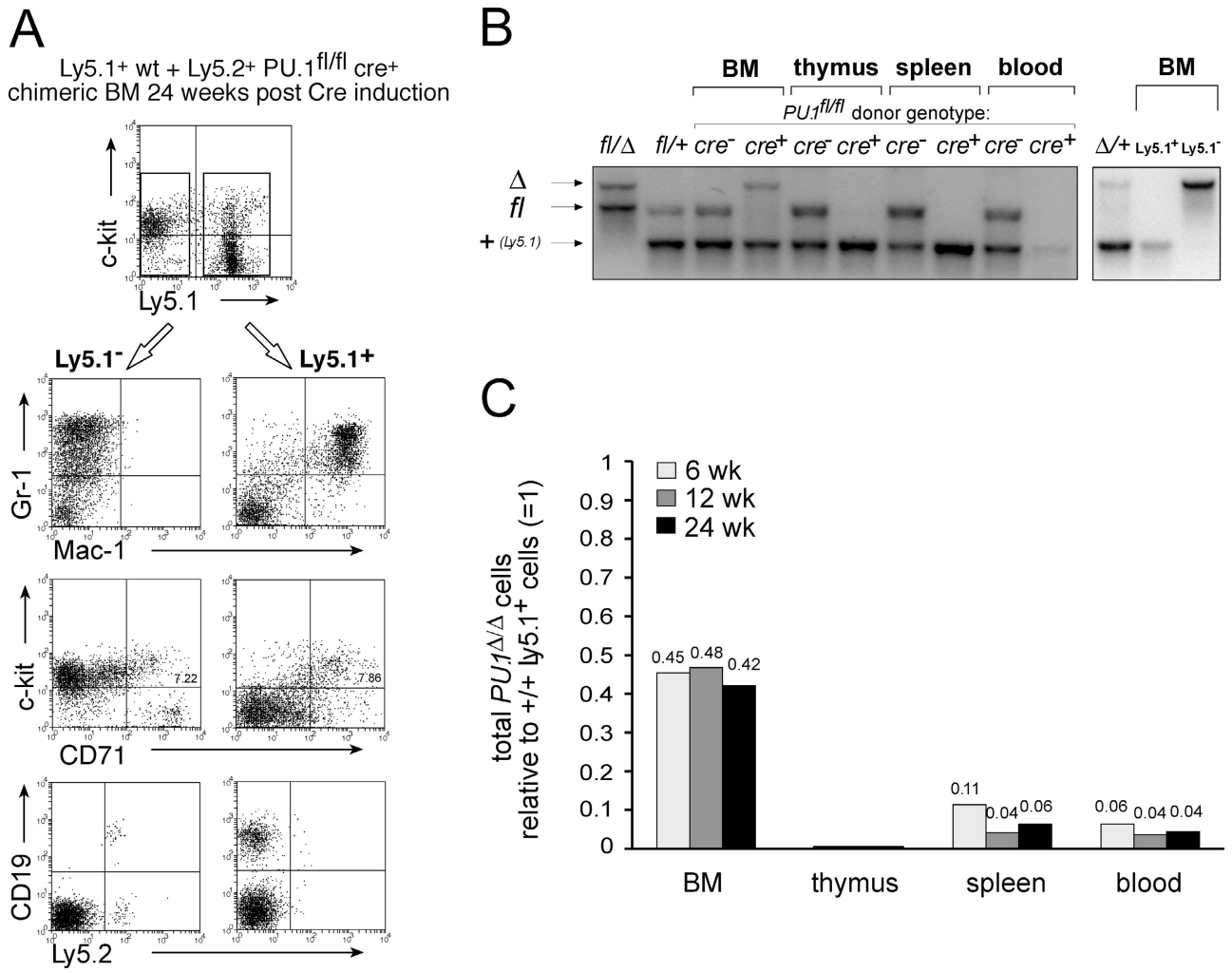

Figure 6. Competitive BM reconstitution. (A) *PU.1*$^{fl/fl}$ MxCre$^+$ BM was mixed with Ly5.1 BM (2:1 ratio) and used to reconstitute lethally irradiated Ly5.1 mice. The degree of reconstitution was assessed from peripheral blood after 6 wk, followed by the induction of *PU.1* deletion by polyIC. Flow cytometric analysis of spleen from chimeric mice at 24 wk. Ly5.1$^-$ cells were almost entirely PU.1-deficient granulocytes (Gr-1$^+$Mac-1$^-$), whereas Ly5.1$^+$ competitors were Gr-1$^+$Mac-1$^+$ (macrophages/granulocytes) and CD19$^+$Ly5.2$^-$ (B cells). c-kit$^+$CD71$^+$ erythroid progenitors were present in both populations. (B) BM, thymus, spleen, and blood cells and sorted Ly5.1$^-$ and Ly5.1$^+$ BM cells were subjected to PCR for *PU.1* exon-5 deletion. *PU.1*$^{fl/\Delta}$, *PU.1*$^{fl/+}$, and *PU.1*$^{+/\Delta}$ tail DNA was used as a control. (+) Competitor Ly5.1$^+$ DNA. Note that the synthesis of *+ (WT)* allele PCR product is more efficient that that of *Δ* allele and cannot be used for relative quantification of host/donor cellular contribution to chimerism. *PU.1*$^{\Delta/\Delta}$ was amplified from the BM, whereas no *PU.1*-deleted cells were found in the thymus, spleen, or blood. (C) The percentage of *PU.1*$^{\Delta/\Delta}$ cells in hematopoietic organs was determined at 6, 12, and 24 wk after polyIC injection. Total *PU.1*$^{\Delta/\Delta}$ cells (Ly5.1$^-$) are shown relative to Ly5.1$^+$ cells. The numbers have been normalized for the predeletion reconstitution frequency. Because cells lose CD45 expression in some lineages, mutant cells were defined as Ter119$^-$Ly5.1$^-$.

PU.1-deficient stem cells do not generate lymphoid progeny

It has been reported that *PU.1*$^{-/-}$ fetal liver stem cells are unable to reconstitute adult hematopoiesis (18). However, given our data showing the abundance of PU.1-deficient cells in adult organs, we reinvestigated this issue using BM chimeras. Ly5.1 mice were reconstituted with *PU.1*$^{fl/fl}$ MxCre$^+$ (Ly5.2) and competing Ly5.1 BM cells. After 6 wk, relative reconstitution was assessed by analysis of peripheral blood. PU.1 inactivation was then induced by polyIC and mice were analyzed at 6, 12, and 24 wk after deletion. Because PU.1 is required for expression of *CD45 (Ly5)* (25, 26) and *Mac-1* (27) in myeloid cells, we used the absence of these markers to determine the efficiency of PU.1 deletion in the reconstituting donor cells (Fig. 6 A). A small number of nondeleted *PU.1*$^{fl/fl}$ Ly5.2$^+$ cells could be distinguished easily from the other populations (Fig. 6 A). PCR analysis on DNA from total hematopoietic tissues or sorted Ly5.1$^-$ (donor) and Ly5.1$^+$ (wild-type competitor) BM cells for *Δ*, *fl*, and *+ (WT)* alleles confirmed the validity of this strategy (Fig. 6 B). Unexpectedly, *PU.1*$^{\Delta/\Delta}$ Ly5.1$^-$ cells generated significant BM cellularity at all time points (Fig. 6 C). Up to 80% of BM Ly5.1$^-$ cells expressed the marker combination Gr-1$^+$c-kit$^+$ and were proliferating; this was indicative of con-

tinuing granulopoiesis. In addition to granulocytes, $CD71^+$c-kit^+ erythroid precursors were detected (Fig. 6 A). On differential count, these $Ly5.1^-$ BM cells had the same increase in blast cells and immature granulocytes as BM of nonchimeric PU.1-deficient mice (unpublished data). PU.1-deficient progenitors also essentially were unable to contribute to T or B lymphopoiesis (Fig. 6 A). PU.1-deficient granulocytes were unable to compete with wild-type cells to repopulate blood and spleen; this effect also was observed in the peripheral blood of nonchimeric PU.1-deficient animals (Table I). Thus, in contrast to reconstitution of irradiated recipients with *PU.1*$^{-/-}$ fetal liver (18) or *PU.1*$^{\Delta/\Delta}$ BM deleted stem cells (unpublished data), PU.1 deletion in steady-state hematopoiesis produced mutant stem cells with long-term erythromyeloid cell production capacity that is biased toward the granulocyte lineage.

DISCUSSION

PU.1 deficiency results in enhanced granulopoiesis

The most striking abnormality of PU.1-deficient BM was the overwhelming granulopoiesis that virtually excluded other lineages. This phenotype was surprising as it contrasted with the lack of granulocytes in *PU.1*$^{-/-}$ fetal livers (16, 17). The cells expressed markers indicative of an immature granulocyte (c-kit, Ly6C) but not the PU.1 target Mac-1 (Fig. 2 A) and appeared to undergo perturbed maturation in vivo (Fig. 3 A). Previous studies on PU.1-deficient fetal liver (28) or neonates (29–31) indicated that the hematopoietic progenitors lacked G-CSF responsiveness ex vivo, but could be grown in IL-3. These studies are complicated by the distinct phenotypes observed in these strains that have not been satisfactory explained (32). The germ-line mutation reported here most resembles the more severe phenotype of Scott et al. (16), in that the mice die at birth and lack granulocytes, whereas the phenotype of McKercher et. al. is relatively less severe and results in postnatal lethality and the development of some immature granulocytes (17). The granulocytes shown here, although displaying some morphologic maturation and expression of G-CSFR and primary and secondary granules, clearly were aberrant in their differentiation in that they maintained c-kit expression. This phenotype was strikingly different than the lack of fetal liver granulocytes, and suggested that in adults, PU.1 suppressed the formation of granulocytic progenitors, and subsequently, was required for their final functional maturation. Heightened in vitro granulocyte response and a corresponding BM neutrophilia also were reported for mice with conditional deletions of *SOCS3* (33) and *STAT3* (34), but in contrast to the *PU.1* mutation, the cells in both of these mutants were hyperresponsive to G-CSF in vitro.

The analysis of clonogenic progenitors from mutant BM and spleen demonstrated that multiple dramatic changes occurred in the absence of PU.1, paralleling the hematologic aberrations that were observed in vivo. These included a marked loss of clonogenic cells that were responsive to GM-CSF or M-CSF. In sharp contrast, granulocyte progenitors occurred at abnormally high frequency in the BM and spleen; this provided a basis for the enhanced granulopoiesis that was observed in these tissues. Although the granulocytic population exhibited full dependency on growth factors for proliferation, the clonogenic cells exhibited several abnormalities, including heightened responsiveness to IFNγ and a failure to respond to IL-6, an agent with actions similar to G-CSF when acting on normal granulocytic progenitor cells. In contrast to granulopoiesis, the loss of cells that were responsive to M-CSF and GM-CSF was identical to that reported previously and is likely due to the induced failure of existing and newly-formed cells to express the cognate receptors, as was noted previously in fetal *PU.1*$^{-/-}$ cells (28). Although GM-CSF and M-CSF are the only known stimuli for macrophage formation in vivo (35), we observed the growth of IL-3–stimulated macrophage colonies from PU.1-deficient cells; this indicated that macrophage development may not be lost. This finding also was supported by the presence of almost normal numbers of BM monocytes 8 wk after the induction of *PU.1* deletion (Table I).

Within the erythroid and megakaryocytic cell lineages, the PU.1-deficient adult phenotype was complex because the pronounced loss of clonogenic cells and nucleated erythrocytes from the BM was mirrored by increased erythromegakaryopoiesis in the mutant spleen (Table III). Such extramedullary hematopoiesis has been observed in other situations in which granulocyte cells become dominant in the BM (36, 37). The presence of erythromegakaryocytic colonies in the spleen contrasted with the loss of responsiveness to EPO + SCF or IL-3 (38) and the premature terminal differentiation (19) that was reported for *PU.1*$^{-/-}$ fetal liver erythroid progenitors; this highlighted another difference between fetal and adult hematopoiesis.

B lymphocyte numbers were reduced, but not eliminated, in the BM after gene inactivation which suggests a role for PU.1 in B lymphopoiesis. However, the interpretation of these results is problematic because polyIC treatment results in a transient-type I IFN response that was demonstrated to reduce BM B cell numbers by >80% (21). After 8 wk, B and T cells were still identifiable in BM and spleen; however, we were unable to determine if these were surviving mature lymphocytes and progeny of their homeostatic proliferation; lymphocytes that were generated from a wave of intermediate precursors, such as those preB cells seen 2 wk after PU.1 deletion; or lymphocytes that developed from early lymphoid progenitors. To better assess lymphoid development, we performed BM reconstitution in the presence of wild-type competitors (Fig. 6). These experiments demonstrate that PU.1-deficient lymphopoiesis was incapable of producing B or T cells in this environment. Therefore, we propose that the decrease in B cells in PU.1-deficient BM reflected a defect in the production of early lymphoid progenitors, potentially via the regulation of the *IL-7Rα* (15) and not a defect in mature B cell survival. This conclusion is supported by the normal development of *PU.1*$^{fl/fl}$ B cells when Cre expression was driven from the

committed B cell–specific *CD19* regulatory sequences (unpublished data). In summary, we propose that PU.1 regulates B and T lymphopoiesis by controlling the commitment of multipotent progenitors and not through the regulation of lymphopoiesis directly.

PU.1 regulates the differentiation of multipotent lymphoid and myeloid progenitors

The enhanced BM granulopoiesis and highly aberrant in vitro clonogenic responses that were observed in the absence of PU.1 suggested that PU.1 regulated the balanced generation of multipotent myeloid progenitors (CMP and GMP) or significantly changed their developmental potential. Analysis of mutant BM indicated that the lin^- compartment contained a uniform population of $c\text{-}kit^+$ cells with blast-like morphology that lacked all other commonly used markers to isolate multipotent progenitor cells prospectively (Fig. 4 A). This observation, combined with the presence of the most primitive blast colony-forming cells in BM (Table II) and spleen (Table III), the enhanced numbers of day 12 CFU-spleen (not depicted) and the ability of $PU.1^{\Delta/\Delta}$ BM to reconstitute granulo- and erythropoiesis for at least 24 wk (Fig. 6), suggested that PU.1 was not required for stem cell function, but for the balanced commitment of the immediate downstream progenitors. In vitro analysis of defined myeloid progenitor populations that were isolated immediately after *PU.1* inactivation supported this model because CMPs gave rise to relatively normal numbers of colonies, whereas GMPs had markedly increased granulocytic potential. Taken together, these data demonstrate that the neutrophilia that was observed in the PU.1-deficient mice arose as a result of deregulated production of GMP-derived granulocytes. A mechanical explanation for this observation could be that after loss of *PU.1*, a switch to granulocytic commitment occurs earlier in the pathway of transient amplifying myeloid progenitors that favors the granulocytic lineage over the monocyte fate. Immediately after *PU.1* deletion, CMPs can still support macrophage development in response to IL-3, whereas this potential is lost prematurely in GMPs (Fig. 5). A switch from macrophage to granulocytic differentiation also has been observed in the absence of the PU.1-interacting partner IRF-8 (IFN consensus sequence-binding protein); this suggests that this interaction also is important in vivo (13, 39).

Recently, it was reported that the targeted deletion of regulatory enhancer sequences that reduced *PU.1* expression by 80% resulted in the development of AML (11). In this model, preleukemic BM had some features that were reminiscent of those reported here, including enhanced granulopoiesis and loss of M-CSFR and GM-CSFR expression. In contrast, these cells continued to express PU.1 targets Mac-1 and FcγRII/III and had normal numbers of G-CSF–responsive progenitors; this suggests that distinct processes are occurring (11). The rapid onset of the neutrophilia (Figs. 2 and 3), the erythromyeloid differentiation capacity of PU.1-deficient stem cells in chimeric mice in the absence of detectable disease (Fig. 6), and the appearance of excess growth factor–dependent granulocyte colonies in vitro shortly after PU.1 deletion (Fig. 5) suggested that in our model, the deregulated differentiation occurred independently of any subsequent transformation event. Our preliminary studies of aging $PU.1^{\Delta/\Delta}$ mice revealed that PU.1-deficient mice do develop myeloid leukemia at a high frequency (unpublished data). However, the resulting leukemias are heterogeneous in terms of their in vitro growth properties and ability to transfer the disease to secondary recipients; this suggests that the role of PU.1 as a tumor suppressor is more complex than currently appreciated (11, 12). In an attempt to provide further molecular insights into the role that PU.1 plays in the multipotent progenitors, we analyzed gene expression in BM lin^- cells. PU.1-deficient progenitors had reduced expression of *M-CSFR*, *GM-CSFRα*, and *IL-7Rα*, but not *G-CSFR*. The normal expression of *G-CSFR* contrasted with the absence of this receptor in $PU.1^{-/-}$ from fetal liver (28, 29, 31), and provided a potential explanation of the increased granulopoiesis. However, $PU.1^{\Delta/\Delta}$ BM also showed increased granulocyte colonies in the presence of IL-3 or IL-3/SCF/EPO which indicated that the expression of G-CSFR was not solely responsible for the phenotype. Moreover, at longer time points after *PU.1* inactivation, the relative contribution of G-CSF in driving the proliferation and differentiation of clonogenic progenitors decreased compared with IL-3/SCF; this suggested that these factors were the more important. Unexpectedly, PU.1 also was required for the expression of the IL-6 and LIF receptor components (*IL-6Rα* and *gp130*) and *IL-5Rα* expression (Fig. 4); this further supported the proposition that PU.1 regulates the response of progenitors to extrinsic regulatory signals. We also assessed the expression of a cohort of key transcriptional regulators of early hematopoiesis, several of which—including *IRF-8* and *GATA-1*—were expressed at lower levels in the absence of PU.1 (Fig. 4 C). In contrast, *C/EBPα*, whose expression is essential for the CMP to GMP transition (40), was increased in $PU.1^{\Delta/\Delta}$ lin^- BM cells (Fig. 4 C). A recent study in $PU.1^{-/-}$ cell lines demonstrated that *C/EBPα* is induced by G-CSF signaling and that the ratio of C/EBPα to PU.1 is crucial to commitment to the granulocyte cell fate, potentially providing a candidate mechanism for the excess granulopoiesis observed in the $PU.1^{\Delta/\Delta}$ tissues (41). G-CSFR expression already is detectable in the HSC (42) which makes this G-CSF-*C/EBPα* proposal in the absence of balancing PU.1 action an even more attractive hypothesis. In addition to these important regulatory genes, it was striking that PU.1 also was required for the expression of several myeloid markers, including Mac-1 (27), FcγRII/III (24), CD45 (25), and F4/80 (Figs. 2–4). This makes interpretation of flow cytometric data problematic without using the in vitro clonogenic progenitor approach that is outlined here.

The prevailing models of PU.1 function in hematopoiesis have been derived almost exclusively from studies of fetal

liver cells where lymphoid versus macrophage lineage commitment depends on the graded levels of PU.1 (15, 43). In contrast, *PU.1* expression in myeloid progenitors was proposed not to be required for cell commitment but for the response to extrinsic signals once a cell is restricted to a particular lineage (28, 29, 44, 45). Although this latter model is consistent with the requirement for PU.1 for the expression of a number of lineage-specific cytokine receptors, the grossly deregulated granulopoiesis; absence of de novo lymphopoiesis; and the loss of discernable CLP, CMP, GMP, or MEP populations along with long-term self-renewing hematopoietic activity suggests that the predominant developmental role of PU.1 in the adult is to control the balanced transition of the stem cell through the progressively committed multipotent progenitor stages.

MATERIALS AND METHODS

Construction and genotyping of PU.1^{gfp} mice. The *PU.1*-targeting construct used the pKW11 vector consisting of a splice acceptor, stop codons in all reading frames, an IRES, *eGFP* cDNA, a SV40 polyadenylation signal, and a *PGK-Neo^r* gene. Genomic DNA containing *LoxP* flanked exon-5 was cloned in front of the pKW11 insert. Homology arms of 2.6 kb (5′) and 3 kb (3′) were amplified from a *PU.1*-containing bacterial artificial chromosome and cloned into the final targeting vector. The linear targeting vector was introduced into the 3′ untranslated region of *PU.1* by homologous recombination in C57BL/6 ES cells. Neomycin-resistant clones were screened by Southern hybridization using 5′ (digested with *Eco RI*, giving wild-type 6.6 kb and *PU.1^{gfp}* 5.3 kb) and 3′ (*Bgl II* wild-type 7.2 kb and *PU.1^{gfp}* 5.4 kb) probes. Targeted ES cell clones were injected into BALB/c blastocysts to obtain chimeric founders. Germ-line transmission was achieved with two clones. PCR genotyping was performed using the primer combination: *a*: 5′-TGGCGCCTACCGGTGGATGTGG-3′, *b*: 5′-CTGTCTGCCACCACCTGCCTACATT-3′, *c*: 5′-GTGCTTCCTTGGGAGTCTGGCGCT-3′, *d*: 5′-GCACACATGCGTGTTTGTGGATGCT-3′, and *e*: 5′-GCAGGCTCGATAGATGCCTTACACC-3′. Primer combination *a/b/c* gave PCR fragments of 680 bp (*b/c* wild type), 512 bp (*a/b PU.1^{gfp}*), and 738 bp (*b/c PU.1^{h}*). Whereas *b/d* gave an additional 837-bp deleted band (*b/d PU.1$^{\Delta}$*), the combination *c/d/e* gave 466 bp (c/e *PU.1^{gfp}*) and 565 (d/e *PU.1$^{\Delta gfp}$*). Deletion frequency was determined by amplification of genomic DNA followed by electrophoresis on a 2% agarose gel. Tail DNA of all genotypes was used as PCR controls in all experiments.

PU.1 conditional deletion and mouse strains. The *PU.1* alleles, *flpe* (46), *MxCre* (20), *Deleter-Cre* (47), and Ly5.1 congenic mouse lines were maintained on a C57BL/6 background. The IRES-GFP-Neo cassette of *PU.1^{gfp}* was deleted in the germline using flpe. Nonconditional *PU.1$^{\Delta}$* mice were created by breeding *PU.1$^{fl/+}$* with *Deleter-Cre*. For conditional inactivation *PU.1$^{fl/fl}$* MxCre$^+$ mice were weighed and injected i.p. with 12.5 μg/g polyIC (1 mg/ml) two times at 3 d apart with the exception of the short-term experiments in Fig. 6, where polyIC was injected 36 h and 24 h before analysis. Deletion frequency was monitored for each experiment using PCR primers that were outlined above. In all experiments, *PU.1$^{fl/fl}$* MxCre$^-$ mice were used as controls for nonspecific polyIC effects. Mice were bred and maintained at the Walter and Eliza Hall Institute under Animal Ethics Committee guidelines.

Analysis of hematologic organs. Peripheral blood cell count and platelet counts were determined using a hemocytometer. Single cell suspensions from femoral BM, spleen, and peritoneal cavity were cytocentrifuged onto slides, stained with May-Grunwald-Giemsa, and differential cell counts were performed. Organs were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin/eosin.

Monoclonal antibodies and flow cytometry. mAbs against the following antigens were used as fluorescent or biotin conjugates for cell staining and sorting: Ly 5.2 (AL1-4A2), Ly5.1 (A20-1.1), Mac-1 (M1/70), Gr-1 (RA6-8C5), FcγRII/III (2.4G2), B220 (RA3-6B2), CD19 (ID3), Ter119, CD4 (GK1.5), and CD8 (YTS 169.4) were purified from hybridoma supernatants and conjugated to fluorochromes. c-kit (2B8), Sca-1 (E13-161-7), IL7Rα (A7R34), CD34 (RAM34), CD71 (C2), and Ly6C (5075–3.6) were from BD Biosciences. Anti–rat immunoglobulin–Texas red, and phycoerythrin- and PerCP-Cy5.5-streptavidin (BD Biosciences) were used as secondary detection reagents. Sorting and analysis was performed on a FACStar-Plus or a DiVa high-speed flow cytometer (BD Biosciences). For cell cycle status, sorted cells were fixed in 70% ethanol overnight, washed, and stained in cell cycle buffer (0.1% sodium acetate, 0.2% Triton X-100, 10 μg/ml RNase A, 50 μg/ml propidium iodide).

Isolation of bone marrow precursor populations. The BM precursor populations were isolated by procedures that were described in detail elsewhere (48). The CMP population was identified as Lin$^-$Sca-1$^-$IL-7Rα$^-$c-kit$^+$CD34$^+$FcγR^{low} cells. GMPs were defined as Lin$^-$Sca-1$^-$IL-7Rα$^-$c-kit$^+$FcγR$^+$CD34$^+$ cells and the MEPs as Lin$^-$Sca-1$^-$IL-7Rα$^-$c-kit$^+$FcγR$^-$CD34$^-$ cells. HSCs were defined as Lin$^-$Sca-1^{hi}IL-7Rα$^-$c-kit^{hi} and CLPs as Lin$^-$Sca-1^{int}IL-7Rα$^+$c-kit^{int} cells.

Western blotting. Total protein extracts were produced from equivalent numbers of cells and Western blotting was performed as described previously (49). Rabbit anti–PU.1 (T21) and goat anti–β-actin (I-19) were obtained from Santa Cruz Biotechnology, Inc.

Bone marrow reconstitution. For long-term competitive assays, lethally irradiated Ly5.1 mice were reconstituted with *PU.1$^{fl/fl}$ MxCre$^+$* BM mixed at 3:1 ratio with Ly5.1 competitor. The degree of reconstitution was determined after 6 wk by analysis of peripheral blood with Ly5.1 and Ly5.2 mAb. PU.1 deletion was induced by polyIC injection as above and the chimeras were analyzed 6, 12, and 24 wk later. Because PU.1 regulates CD45 in some lineages, donor cells were defined as Ly5.1$^-$ after erythroid (Ter119$^+$) cells were excluded.

In vitro clonogenic cultures. BM (2.5×10^4) and spleen (5×10^4) cells were cultured in 1 ml of Dulbecco's modified Eagle medium containing 0.3% agar and analyzed as described previously (50). Sorted granulocytes (Gr-1$^+$) were similarly cultured in liquid medium plus G-CSF for 7 d. The recombinant cytokines were used at the following final concentrations GM-CSF, G-CSF, IL-3, M-CSF, LIF (10 ng/ml), SCF and IL-6 (100 ng/ml), flt3L (500 ng/ml), IFNγ (2×10^3 U/ml), thrombopoietin (50 ng/ml), and EPO (2I U/ml). After 7 d of incubation, differential colony counts were performed on fixed whole mount preparations that were stained for acetylcholinesterase, Luxol fast blue, and hematoxylin.

RT-PCR analysis. Semi-quantitative RT-PCR was performed as previously described (51). For each primer set, a sample of the PCR reaction was taken at three cycle numbers; each sample was four cycles apart. Amplification products all spanned introns and were visualized on 2% agarose gels. Primer sequences are available upon request.

We thank J. Carneli, J. Merryfull, and K. Gray for animal husbandry; J. Brady, A. D'Amico, W. Cook, and J. Werzowa for advice and assistance; M. Busslinger for reagents; and J. Adams for helpful comments on the text.

This work was supported by the Cancer Council Victoria, the National Institutes of Health, Bethesda (grant no. CA22556), and The National Health and Medical Research Council of Australia. S.L. Nutt is supported by The Walter and Eliza Hall Institute Metcalf Fellowship.

The authors have no conflicting financial interests.

Submitted: 7 January 2005
Accepted: 17 March 2005

REFERENCES

1. Kondo, M., A.J. Wagers, M.G. Manz, S.S. Prohaska, D.C. Scherer, G.F. Beilhack, J.A. Shizuru, and I.L. Weissman. 2003. Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu. Rev. Immunol.* 21:759–806.
2. Akashi, K., D. Traver, T. Miyamoto, and I.L. Weissman. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature.* 404:193–197.
3. Kondo, M., I.L. Weissman, and K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell.* 91:661–672.
4. Traver, D., T. Miyamoto, J. Christensen, J. Iwasaki-Arai, K. Akashi, and I.L. Weissman. 2001. Fetal liver myelopoiesis occurs through distinct, prospectively isolatable progenitor subsets. *Blood.* 98:627–635.
5. Mebius, R.E., T. Miyamoto, J. Christensen, J. Domen, T. Cupedo, I.L. Weissman, and K. Akashi. 2001. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3− cells, as well as macrophages. *J. Immunol.* 166:6593–6601.
6. Kawamoto, H., T. Ikawa, K. Ohmura, S. Fujimoto, and Y. Katsura. 2000. T cell progenitors emerge earlier than B cell progenitors in the murine fetal liver. *Immunity.* 12:441–450.
7. Ichikawa, M., T. Asai, T. Saito, G. Yamamoto, S. Seo, I. Yamazaki, T. Yamagata, K. Mitani, S. Chiba, H. Hirai, et al. 2004. AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. *Nat. Med.* 10:299–304.
8. Mikkola, H.K., J. Klintman, H. Yang, H. Hock, T.M. Schlaeger, Y. Fujiwara, and S.H. Orkin. 2003. Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature.* 421:547–551.
9. Lloberas, J., C. Soler, and A. Celada. 1999. The key role of PU.1/SPI-1 in B cells, myeloid cells and macrophages. *Immunol. Today.* 20:184–189.
10. Nutt, S.L., D. Metcalf, A. D'Amico, M. Polli, and L. Wu. 2005. Dynamic regulation of PU.1 expression in multipotent hematopoietic progenitors. *J. Exp. Med.* 201:221–231.
11. Rosenbauer, F., K. Wagner, J.L. Kutok, H. Iwasaki, M.M. Le Beau, Y. Okuno, K. Akashi, S. Fiering, and D.G. Tenen. 2004. Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *Nat. Genet.* 36:624–630.
12. Cook, W.D., B.J. McCaw, C. Herring, D.L. John, S.J. Foote, S.L. Nutt, and J.M. Adams. 2004. PU.1 is a suppressor of myeloid leukemia, inactivated in mice by gene deletion and mutation of its DNA binding domain. *Blood.* 104:3437–3444.
13. Marecki, S., and M.J. Fenton. 2000. PU.1/Interferon Regulatory Factor interactions: mechanisms of transcriptional regulation. *Cell Biochem. Biophys.* 33:127–148.
14. Zhang, D.E., S. Hohaus, M.T. Voso, H.M. Chen, L.T. Smith, C.J. Hetherington, and D.G. Tenen. 1996. Function of PU.1 (Spi-1), C/EBP, and AML1 in early myelopoiesis: regulation of multiple myeloid CSF receptor promoters. *Curr. Top. Microbiol. Immunol.* 211:137–147.
15. DeKoter, R.P., H.J. Lee, and H. Singh. 2002. PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. *Immunity.* 16:297–309.
16. Scott, E.W., M.C. Simon, J. Anastasi, and H. Singh. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science.* 265:1573–1577.
17. McKercher, S.R., B.E. Torbett, K.L. Anderson, G.W. Henkel, D.J. Vestal, H. Baribault, M. Klemsz, A.J. Feeney, G.E. Wu, C.J. Paige, and R.A. Maki. 1996. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J.* 15:5647–5658.
18. Scott, E.W., R.C. Fisher, M.C. Olson, E.W. Kehrli, M.C. Simon, and H. Singh. 1997. PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors. *Immunity.* 6:437–447.
19. Back, J., A. Dierich, C. Bronn, P. Kastner, and S. Chan. 2004. PU.1 determines the self-renewal capacity of erythroid progenitor cells. *Blood.* 103:3615–3623.
20. Kuhn, R., F. Schwenk, M. Aguet, and K. Rajewsky. 1995. Inducible gene targeting in mice. *Science.* 269:1427–1429.
21. Lin, Q., C. Dong, and M.D. Cooper. 1998. Impairment of T and B cell development by treatment with a type I interferon. *J. Exp. Med.* 187:79–87.
22. Suzuki, S., A. Kumatori, I.A. Haagen, Y. Fujii, M.A. Sadat, H.L. Jun, Y. Tsuji, D. Roos, and M. Nakamura. 1998. PU.1 as an essential activator for the expression of gp91(phox) gene in human peripheral neutrophils, monocytes, and B lymphocytes. *Proc. Natl. Acad. Sci. USA.* 95:6085–6090.
23. Chen, H., D. Ray-Gallet, P. Zhang, C.J. Hetherington, D.A. Gonzalez, D.E. Zhang, F. Moreau-Gachelin, and D.G. Tenen. 1995. PU.1 (Spi-1) autoregulates its expression in myeloid cells. *Oncogene.* 11: 1549–1560.
24. Feinman, R., W.Q. Qiu, R.N. Pearse, B.S. Nikolajczyk, R. Sen, M. Sheffery, and J.V. Ravetch. 1994. PU.1 and an HLH family member contribute to the myeloid-specific transcription of the Fc gamma RI-IIA promoter. *EMBO J.* 13:3852–3860.
25. Anderson, K.L., S.L. Nelson, H.B. Perkin, K.A. Smith, M.J. Klemsz, and B.E. Torbett. 2001. PU.1 is a lineage-specific regulator of tyrosine phosphatase CD45. *J. Biol. Chem.* 276:7637–7642.
26. Medina, K.L., J.M. Pongubala, K.L. Reddy, D.W. Lancki, R. Dekoter, M. Kieslinger, R. Grosschedl, and H. Singh. 2004. Assembling a gene regulatory network for specification of the B cell fate. *Dev. Cell.* 7:607–617.
27. Pahl, H.L., R.J. Scheibe, D.E. Zhang, H.M. Chen, D.L. Galson, R.A. Maki, and D.G. Tenen. 1993. The proto-oncogene PU.1 regulates expression of the myeloid-specific CD11b promoter. *J. Biol. Chem.* 268: 5014–5020.
28. DeKoter, R.P., J.C. Walsh, and H. Singh. 1998. PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors. *EMBO J.* 17:4456–4468.
29. Anderson, K.L., K.A. Smith, K. Conners, S.R. McKercher, R.A. Maki, and B.E. Torbett. 1998. Myeloid development is selectively disrupted in PU.1 null mice. *Blood.* 91:3702–3710.
30. Anderson, K.L., K.A. Smith, F. Pio, B.E. Torbett, and R.A. Maki. 1998. Neutrophils deficient in PU.1 do not terminally differentiate or become functionally competent. *Blood.* 92:1576–1585.
31. Anderson, K.L., K.A. Smith, H. Perkin, G. Hermanson, C.G. Anderson, D.J. Jolly, R.A. Maki, and B.E. Torbett. 1999. PU.1 and the granulocyte- and macrophage colony-stimulating factor receptors play distinct roles in late-stage myeloid cell differentiation. *Blood.* 94:2310–2318.
32. Fisher, R.C., and E.W. Scott. 1998. Role of PU.1 in hematopoiesis. *Stem Cells.* 16:25–37.
33. Croker, B.A., D. Metcalf, L. Robb, W. Wei, S. Mifsud, L. DiRago, L.A. Cluse, K.D. Sutherland, L. Hartley, E. Williams, et al. 2004. SOCS3 is a critical physiological negative regulator of G-CSF signaling and emergency granulopoiesis. *Immunity.* 20:153–165.
34. Lee, C.K., R. Raz, R. Gimeno, R. Gertner, B. Wistinghausen, K. Takeshita, R.A. DePinho, and D.E. Levy. 2002. STAT3 is a negative regulator of granulopoiesis but is not required for G-CSF-dependent differentiation. *Immunity.* 17:63–72.
35. Metcalf, D., T.A. Willson, D.J. Hilton, L. Di Rago, and S. Mifsud. 1995. Production of hematopoietic regulatory factors in cultures of adult and fetal mouse organs: measurement by specific bioassays. *Leukemia.* 9:1556–1564.
36. de Haan, G., M. Loeffler, and W. Nijhof. 1992. Long-term recombinant human granulocyte colony-stimulating factor (rhG-CSF) treatment severely depresses murine marrow erythropoiesis without causing an anemia. *Exp. Hematol.* 20:600–604.
37. Holtschke, T., J. Lohler, Y. Kanno, T. Fehr, N. Giese, F. Rosenbauer, J. Lou, K.P. Knobeloch, L. Gabriele, J.F. Waring, et al. 1996. Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. *Cell.* 87:307–317.
38. Fisher, R.C., W.B. Slayton, C. Chien, S.M. Guthrie, C. Bray, and E.W. Scott. 2004. PU.1 supports proliferation of immature erythroid progenitors. *Leuk. Res.* 28:83–89.

39. Scheller, M., J. Foerster, C.M. Heyworth, J.F. Waring, J. Lohler, G.L. Gilmore, R.K. Shadduck, T.M. Dexter, and I. Horak. 1999. Altered development and cytokine responses of myeloid progenitors in the absence of transcription factor, interferon consensus sequence binding protein. *Blood*. 94:3764–3771.
40. Zhang, P., J. Iwasaki-Arai, H. Iwasaki, M.L. Fenyus, T. Dayaram, B.M. Owens, H. Shigematsu, E. Levantini, C.S. Huettner, J.A. Lekstrom-Himes, et al. 2004. Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. *Immunity*. 21:853–863.
41. Dahl, R., J.C. Walsh, D. Lancki, P. Laslo, S.R. Iyer, H. Singh, and M.C. Simon. 2003. Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nat. Immunol*. 4:1029–1036.
42. McKinstry, W.J., C.L. Li, J.E. Rasko, N.A. Nicola, G.R. Johnson, and D. Metcalf. 1997. Cytokine receptor expression on hematopoietic stem and progenitor cells. *Blood*. 89:65–71.
43. DeKoter, R.P., and H. Singh. 2000. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science*. 288: 1439–1441.
44. Henkel, G.W., S.R. McKercher, P.J. Leenen, and R.A. Maki. 1999. Commitment to the monocytic lineage occurs in the absence of the transcription factor PU.1. *Blood*. 93:2849–2858.
45. McKercher, S.R., G.W. Henkel, and R.A. Maki. 1999. The transcription factor PU.1 does not regulate lineage commitment but has lineage-specific effects. *J. Leukoc. Biol*. 66:727–732.
46. Farley, F.W., P. Soriano, L.S. Steffen, and S.M. Dymecki. 2000. Widespread recombinase expression using FLPeR (flipper) mice. *Genesis*. 28:106–110.
47. Schwenk, F., U. Baron, and K. Rajewsky. 1995. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res*. 23:5080–5081.
48. D'Amico, A., and L. Wu. 2003. The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing Flt3. *J. Exp. Med*. 198:293–303.
49. Rosenbauer, F., A. Kallies, M. Scheller, K.P. Knobeloch, C.O. Rock, M. Schwieger, C. Stocking, and I. Horak. 2002. Disabled-2 is transcriptionally regulated by ICSBP and augments macrophage spreading and adhesion. *EMBO J*. 21:211–220.
50. Metcalf, D., L. Di Rago, and S. Mifsud. 2002. Synergistic and inhibitory interactions in the in vitro control of murine megakaryocyte colony formation. *Stem Cells*. 20:552–560.
51. Nutt, S.L., S. Vambrie, P. Steinlein, Z. Kozmik, A. Rolink, A. Weith, and M. Busslinger. 1999. Independent regulation of the two Pax5 alleles during B-cell development. *Nat. Genet*. 21:390–395.