PU.1 is not strictly required for B cell development and its absence induces a B-2 to B-1 cell switch

Min Ye, Olga Ermakova, and Thomas Graf

Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY 10461

In this paper, we describe the unexpected outgrowth of B lineage cells from PU.1$^{-/-}$ fetal liver cultures. The cells express all early B cell genes tested, including the putative PU.1 target genes IL-7R and EBF but not B220, and can produce immunoglobulin M. However, we observed a delay in the PU.1$^{-/-}$ B cell outgrowth and reduced precursor frequencies, indicating that although PU.1 is not strictly required for B cell commitment, it facilitates B cell development. We also ablated PU.1 in CD19-expressing B lineage cells in vivo, using a Cre-lox approach that allows them to be tracked. PU.1 excision resulted in a shift from B-2 cells to B-1–like cells, which dramatically increased with the age of the mice. Our data indicate that this shift is predominantly caused by a B-2 to B-1 cell reprogramming. Furthermore, we found that B-2 cells express substantially more PU.1 than B-1 cells, which is consistent with the idea that maintenance of the B-2 cell phenotype requires relatively high levels of PU.1, but B-1 cells require little.

CORRESPONDENCE
Thomas Graf:
graf@aecom.yu.edu
OR
Min Ye:
ymin@aecom.yu.edu

Abbreviations used in this paper: BCR, B cell receptor; E, embryonic day; MZB, marginal zone B; SCF, stem cell factor.

Mice lacking PU.1 contain neither B cells nor myelomonocytic cells and fetal liver suspensions neither generate B cells nor macrophages in culture (1–3), although immature myeloid cell lines can be established in the presence of IL-3, IL-6, and stem cell factor (SCF) (4). Failure to produce B lineage cells in culture can be rescued by putative downstream target genes: enforced expression of the EBF transcription factor led to B cell outgrowth within 4–6 d, and of IL-7R within 10–14 d and at low frequencies. The cells exhibit immunoglobulin rearrangements and are CD45/B220 antigen negative (5, 6). These observations, together with evidence indicating that PU.1 directly regulates the IL-7R (5) and EBF genes (7), led to the conclusion that PU.1 is required for the commitment of B lineage cells. However, the exact point in development where PU.1 is required is poorly understood. One study described the virtual absence of HSCs (Lin$^-$Sca-1$^+$Kit$^+$ cells) in PU.1$^{-/-}$ fetal liver (8), but another one reported the absence of early B cell precursors (IL-7R$^+$Kit$^+$ cells) (7). It also remains unknown whether PU.1 plays a role at later stages of B cell differentiation, where the gene is also expressed (9).

There are three main subpopulations of B cells in mice and humans: B-1, B-2 (also called follicular), and marginal zone B (MZB) cells. B-1 cells, which exist as B-1a (CD5$^+$) and B-1b (CD5$^-$) variants, are mostly present in the peritoneum where they are maintained by self-renewal. B-1a cells and part of B-1b cells are thought to be of fetal origin (10). In contrast, B-2 cells reside in follicles of secondary lymphoid organs and are replenished from precursors formed in the bone marrow (11). Finally, MZB cells are localized in the marginal zones of splenic follicles and are thought to be recruited from mature B-2 cells (12). Whereas B-2 and MZB cells express B220 antigen, B-1 cells are B220$^{neg/low}$ and express CD43. In addition, the three B cell subsets can be distinguished by the expression of IgM, IgD, CD21, and CD23 cell surface antigens.

Whether B-1 cells represent a lineage distinct from B-2 cells remains controversial (13). The ratio of B-1 and B-2 lineage cells can be dramatically altered by both loss and gain of function experiments. Thus, mice lacking molecules necessary for the initiation of B cell receptor signaling show a loss of B-1 lineage cells and mice expressing specific B cell receptor (BCR)

O. Ermakova's present address is Mouse Biology Unit, European Molecular Biology Laboratory, I-00016 Monterotondo-Scalo, Rome, Italy.

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transgenes show an expansion of the B-2 cell compartment (14). Interestingly, BCR signaling strength determines the ratio between the two cell compartments: in BCR-deficient mice transgenic for LMP2, a BCR surrogate, high LMP2 levels induce the expansion of B-1 type cells whereas low levels promote the formation of B-2 and MZB cells (15). To interpret these results, two explanations were offered. First that B-1 and B-2 cells represent two distinct lineages, where low BCR signaling drives the progression of B-2 progenitors and high signaling drives B-1 cell progression. And second, that the strength of BCR signaling determines which of the two B cell subtypes is specified from a "B-0" cell.

In this paper, we describe the unexpected outgrowth of B lineage cells in culture from PU.1$^{-/-}$ fetal liver. These cells share several markers with B-1 cells. In addition, in vivo deletion of PU.1 in CD19-expressing B lineage cells showed the presence of cells resembling B-1 cells and the concomitant loss of B-2 cells, which is an effect that was more pronounced in older mice. Our data indicate that this imbalance is caused by a B-2 to B-1 cell reprogramming. The detection of the complex alterations caused by PU.1 ablation in vivo was greatly facilitated by a genetic approach that allowed tracking of individual cells containing the deletion.

RESULTS

PU.1-defective fetal liver cultures give rise to B lineage cells

To analyze the role of PU.1 in B cell development we used a recently developed mouse line in which exons 4 and 5, encoding the DNA binding and PEST domains, are flanked by loxP sites. Crossing these mice with a β-actin–Cre line resulted in mice with a germline excision of PU.1 (Fig. 1 A). The homozygous animals produce no detectable PU.1 mRNA, die soon after birth, and their fetal livers contained no detectable Mac-1^{+}Gr1^{+} myelomonocytic cells or B220^{+} CD19^{+} B cells (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20051089/DC1). PU.1$^{-/-}$ liver cell suspensions that were seeded under conditions permissive for both B and myeloid cell growth (S17 stroma cells containing IL-3, IL-7, SCF, and Flt-3L) developed into foci of small round, refractile cells within 2–3 wk from all 21 fetal livers (embryonic day [E] 14.5–18.5) tested (Fig. 1 B). The cultures could be split every 3–4 d, proliferating slightly faster than WT cells, and grew for >6 mo without a detectable crisis. These cells expressed CD19, CD43, CD24 (HSA), and BP1, but not B220 and CD45 antigens. They were also negative for erythroid (Ter119), T cell (Thy-1, CD3, CD4, and CD8), NK cell (NK1.1) and myelomonocytic (Mac-1, Gr1, and F4/80) surface markers (Fig. 1 C and not depicted). Thus, the cultured PU.1$^{-/-}$ cells resemble pro/pre–B cells and also share characteristics with B-1 cells (B220$^{neg/low}$CD43^{+}CD19^{+}). Next, we compared the gene expression profiles (RT-PCR) of freshly isolated fetal liver with cultured derivatives. As can be seen in the left panel of Fig. 1 D, E15.5 PU.1$^{-/-}$ fetal liver cells expressed no B cell specific genes, except E2A (E47). In contrast, cultured PU.1$^{-/-}$ B lineage cells expressed all B cell–specific genes

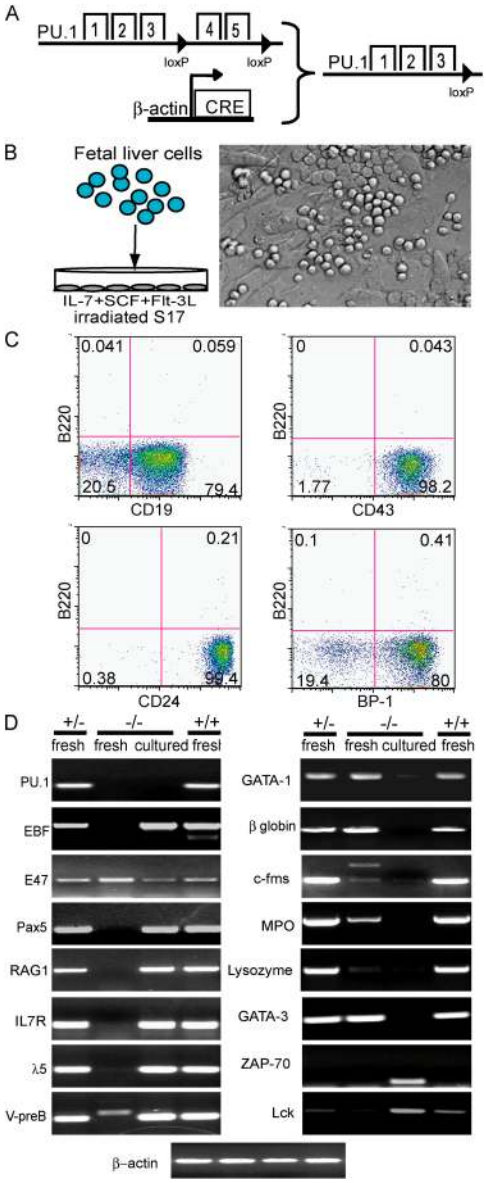

Figure 1. Establishment of B cell cultures from PU.1-deficient fetal liver. (A) Strategy to generate PU.1 germline deletion mice. (B) Schematic of the cell culture system and micrograph of an E14.5 PU.1$^{-/-}$ fetal liver–derived colony growing out after 14 d. (C) FACS analysis of the cultured cells. Numbers in quadrants, set by staining with appropriate isotype controls, represent percentage of cells. (D) RT-PCR analysis of freshly isolated PU.1$^{-/-}$ and control E15.5 fetal liver cells or the knockout cells after culturing.

tested, including EBF and Pax-5 as well as their target genes RAG1, IL-7R, λ5, and V–pre-B, indicating that cultures derived from PU.1$^{-/-}$ fetal liver correspond to bona fide B lineage cells. We further tested whether these PU.1$^{-/-}$ B cells expressed other lineage genes. As shown in the right panel of Fig. 1 D, they were negative for the myeloid genes c-fms (CSF-1R), lysozyme, MPO, and the erythroid genes GATA-1 and β-globin. However, although the cultured PU.1$^{-/-}$ cells did not express the T cell genes GATA-3 or pre-Tα (unpublished data), they expressed lck and ZAP-70,

genes that are normally expressed in T and NK cells and whose products play important roles in TCR signaling. Lck is also expressed in B-1 cells where it regulates BCR signaling transduction (16). Together with the pattern of cell surface antigens detected by FACS on PU.1$^{-/-}$ cells, the gene expression profile suggests that cultured B lineage cells from PU.1$^{-/-}$ fetal liver resemble B-1 cells.

PU.1-defective B cells contain immunoglobulin rearrangements and can express IgM

To examine PU.1$^{-/-}$ fetal liver derived cell cultures for immunoglobulin heavy chain rearrangements by PCR, a series of 5′ primers specific to different V or D region families were used together with a 3′ primer downstream of JH4 (17). Multiple heavy chain DJ and VDJ rearrangements indicated by different sizes of PCR products were found in cultured PU.1$^{-/-}$ fetal liver cells (Fig. 2 A and Table I), but none were detected in freshly isolated PU.1$^{-/-}$ fetal livers (not depicted). Joining of V to D region sequences was seen with both proximal (VH7183) and distal V family genes (VHJ558) within the 21 VDJ rearrangements sequenced (Table I). This suggests that the lack of PU.1 does not prevent a "contraction" of the heavy chain locus as has been described for pro–B cells lacking Pax5 (18). Out of the 11 PU.1$^{-/-}$ B cell lines analyzed, 4 were found to contain low proportions of IgM-positive cells (Fig. 2 B), whereas 7 were negative. To determine whether the negative cells can upregulate IgM expression, one line was grown in the absence of IL-7 and found to become IgM$^+$ (Fig. 2 C). Therefore, cultured PU.1$^{-/-}$ fetal liver derived B cells exhibit heavy chain DJ and VDJ rearrangements and can produce IgM.

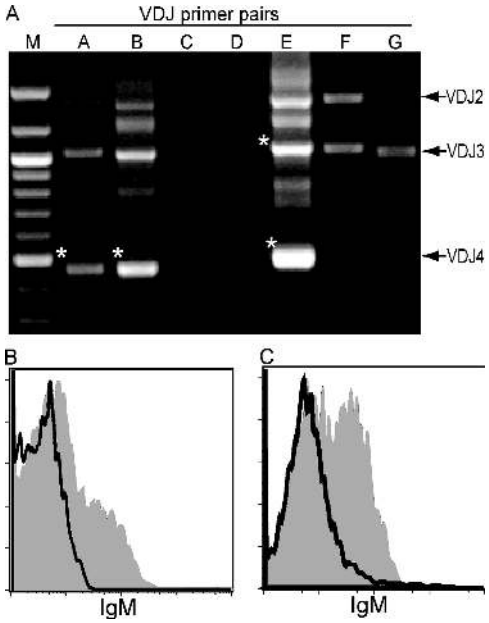

Figure 2. Immunoglobulin rearrangements and IgM expression in cultured PU.1$^{-/-}$ fetal liver cells. (A) Heavy chain (IgH) VDJ rearrangement detected by the use of 7 V_H family consensus primers and one J_H consensus primer (lanes A–G). Asterisks indicate VDJ bands whose nature was confirmed by sequencing (Table I). (B) Surface IgM expression on PU.1$^{-/-}$ clone E18-6.1 (gray shading) compared with isotype control (bold line). (C) PU.1$^{-/-}$ clone E18-6.2 grown in the absence of IL-7 for 7 d (gray shading) compared with isotype control (bold line).

PU.1$^{-/-}$ fetal livers contain fewer B cell precursors, and these are delayed in their outgrowth

To analyze the frequency of B lineage progenitors in PU.1$^{-/-}$ fetal livers, we performed a limiting dilution analysis. Lin$^{-/low}$ Sca-1$^+$c-Kit$^+$ (LSK) cells were sorted from fetal livers of E16.5 PU.1$^{-/-}$ embryos, with a pool of similarly sorted PU.1$^{+/-}$ (het) and WT embryos serving as positive controls. The two cell samples were then placed at increasing cell numbers in wells containing S17 stroma cells plus cytokines and colonies scored between 4 and 22 d. B cell colonies were identified by morphology and confirmed by CD19 antibody staining. The LSK cells from PU.1$^{-/-}$ fetal livers were found to generate ~25 times fewer B cell colonies than the WT/het controls (Fig. 3 A). In addition, the formation of B cell colonies was delayed compared with the controls (Fig. 3 B). These data show that PU.1$^{-/-}$ fetal livers contain a reduced number of B cell progenitors compared with controls and that those that are present show a delayed development into B lineage cells.

In vivo inactivation of PU.1 in CD19$^+$ cells leads to a decrease of B cells in the bone marrow

To determine the role of PU.1 in B cell development in vivo, we crossed the PU.1 conditional knockout mice with a strain that expresses Cre-recombinase under the control of the endogenous CD19 locus (19). To monitor the deletion frequency of PU.1, these mice were then crossed with ROSA26-EYFP reporter mice, in which removal of a LoxP-flanked stop cassette irreversibly activates EYFP expression. The conditional knockout mice have the genotype PU.1$^{-/loxP}$ CD19Cre$^{+/ki}$ROSA26-EYFP$^{+/ki}$ whereas the control mice are PU.1$^{+/loxP}$CD19Cre$^{+/ki}$ROSA26-EYFP$^{+/ki}$, which corresponds in their deleted forms to PU.1 homozygous and heterozygous mice, respectively (Fig. 4 A and Fig. S2 [available at

Table I. Immunoglobulin heavy chain DJ and VDJ rearrangements in PU.1$^{-/-}$ fetal liver–derived B cells

	5′ primer (corresponding families)	No. of clones sequenced	No. of unique clones	No. of clones with in-frame rearrangements
Fetal liver DJ	D_HQ52 (DHQ52)	5	2	1
	DSF(D_HFL16; D_HSP2)	13	4	1
Fetal liver VDJ	V_HA (V_HJ558)	4	2	2
	V_HB (V_HQ52)	5	2	2
	V_HE (V_H7183; V_HDNA4)	9	4	0
	V_HF (V_HJ606; V_HS107; V_HX24)	3	3	0

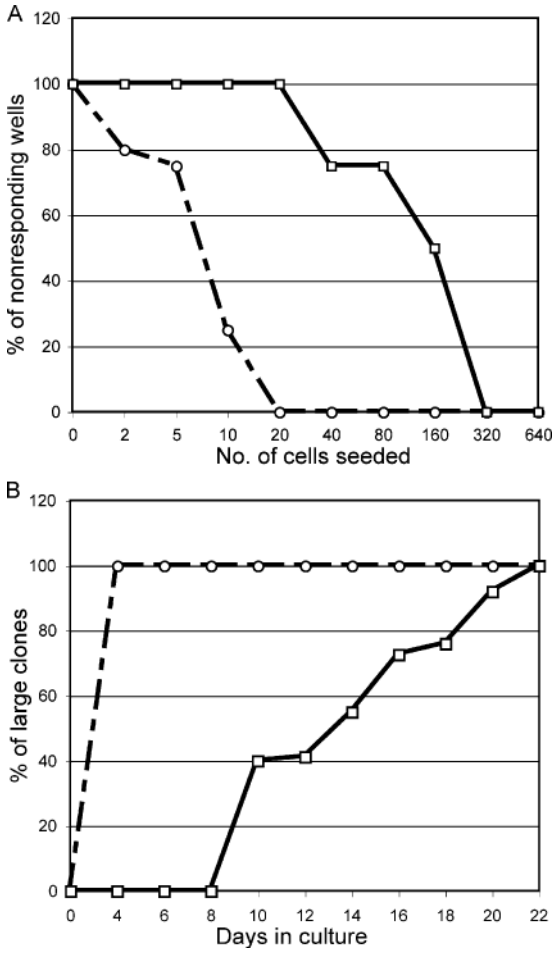

Figure 3. Frequency and outgrowth kinetics of PU.1$^{-/-}$ fetal liver B cells. LSK cells were sorted from E16.5 PU.1$^{-/-}$ as well as a mixture of PU.1$^{+/+}$ with PU.1$^{+/-}$ fetal liver cells and plated in 96-well plates containing S17 cells plus cytokines. Formation of B cell colonies, consisting of >10 cells, was scored between 4 and 22 d of culturing. (A) Percentage of wells with no colonies scored at day 22 relative to cell number seeded. (B) Percentage of large colonies (consisting of >100 cells) scored at different days of culture.

http://www.jem.org/cgi/content/full/jem.20051089/DC1]). PCR analysis of PU.1 DNA in B cells showed that there was an excellent correlation between the activation of EYFP and PU.1 deletion (Fig. 4 B). As shown in Fig. 4 C, the proportion of CD19$^+$ B cells in the bone marrow of the knockout mice was reduced compared with the control group. To examine whether deletion of PU.1 caused a selective loss of early progenitors, we determined the proportion of EYFP$^+$ cells in pro/pre–B cells (CD19$^+$IgM$^-$) and in immature B cells (CD19$^+$IgM$^+$). As illustrated in Fig. 4 (C and D), compared with controls, these values were ~50% lower for pro/pre–B cells in the knockout mice, ~20% lower for immature B cells, and essentially unchanged for B220$^+$CD19$^+$IgD$^+$ mature B cells in the spleen and blood. This indicates that early B cell precursors are selectively lost after PU.1 ablation, probably because they acquire a B-1–like phenotype and leave the bone marrow (see Discussion).

Ablation of PU.1 induces an age-dependent formation of B220^{low}CD43$^+$ cells in the bone marrow, spleen, and blood

As shown in Fig. 5 A, B220^{low} EYFP$^+$ cells were detected in the bone marrow, spleen and blood of the conditional PU.1 knockout mice, and these cells also expressed high levels of CD43. Such cells were not seen in control mice (B220^{low}EYFP$^+$ cells in the spleen represent plasma cells since they are CD19$^-$CD138$^+$EYFP^{bright}, whereas B220^{low} EYFP$^+$ cells from knockouts are CD19$^+$CD138$^-$EYFP$^+$; Fig. 5 A and not depicted). Among the EYFP$^+$ cells with the PU.1 deletion, the expression of B220 varied between the three tissues. Although B220 expression was down-regulated 29.0 ± 3.8-fold in the spleen and 38.1 ± 8.9-fold in the blood, there was only a 12.0 ± 0.6-fold decrease in the bone marrow. Surprisingly, CD45 was only slightly down-regulated in the B220^{low}EYFP$^+$ cells from spleen and blood (1.6 ± 0.3 in spleen and 1.6 ± 0.2 in blood; five knockout mice, 6 mo old) without showing changes in the bone marrow. In addition, as shown in Fig. 5 (B and C), the proportion of B220^{low}EYFP$^+$ cells in the knockouts increased with the age of the mice. As shown in Fig. 5 B, the analysis of peripheral blood of a single mouse over time showed an eightfold increase of B220^{low}EYFP$^+$ cells. In summary, mice with a B cell–specific PU.1 knockout contain CD19$^+$ cells that are B220^{low}CD43$^+$, resembling the PU.1$^{-/-}$ fetal liver–derived B lineage cells. These B-1–like cells increased in number over time.

PU.1 conditional knockouts exhibit an increased ratio of B-1/B-2 cells in the spleen

To analyze the effect of PU.1 deletion in B-2, B-1, and MZB cell subsets, we tested the distribution of cells expressing CD23 and CD21 antigens within the CD19$^+$ B cell population of the spleen from the control and knockout mice. As shown in Fig. 6 (A and B), the distribution of the B-2 cell subset (CD23$^+$CD21$^+$), B-1/transitional B subset (CD23^{low}CD21^{low}), and the MZB subset (CD23$^+$CD21^{high}) varied between the two mouse strains. Whereas 6-mo-old controls contained about threefold more B-2 cells than B-1/transitional B cells (3.0 ± 0.8, four mice, 6 mo old), knockout mice contained about twofold more B-1 than B-2 cells (2.1 ± 0.5, five knockout mice, 6 mo old; B-1 cells were distinguished from transitional B cells by the expression of CD43 and lack of B220 expression; Figs. 5 A and 6 B). In contrast, the proportion of MZB cells, although highly variable between mice, did not differ substantially between the two groups. However, the knockout MZB cells exhibited a small but notable decrease in the expression of CD21 (1.5 ± 0.2, four control mice and five knockout, 6 mo old). The increase of B-1–like cells became more dramatic with age: nearly all B cells of a 14-mo-old conditional knockout were CD23^{low}CD21^{low} and both the B-2 and MZB subsets had disappeared. The analysis of spleen sections from 6-mo-old littermates revealed that the B220^{low}EYFP$^+$ (knockout) cells localized to the follicular region (Fig. 6 C). In contrast, in a 14-mo-old knockout, the spleen structure was completely

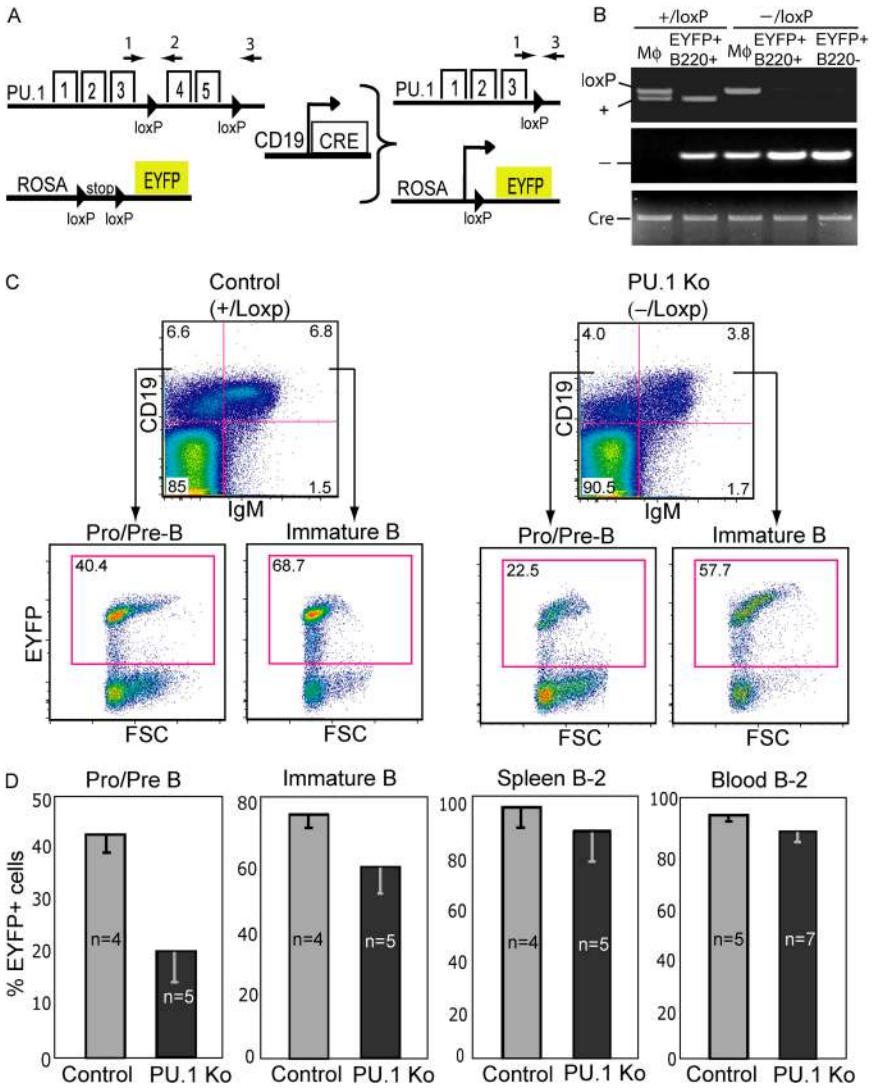

Figure 4. Conditional in vivo PU.1 ablation strategy and analysis of B cells in the bone marrow. (A) Strategy of generating B cell–specific PU.1 knockout mice, and how EYFP expression tracks Cre activity in individual cells. (B) PCR analysis of floxed PU.1 allele deletion in PU.1 conditional knockout mice. DNA was extracted from sorted splenic B cells ($CD19^+EYFP^+B220^+$ cells from $PU.1^{+/loxP}$ control mice; $CD19^+EYFP^+B220^+$ and $CD19^+EYFP^+B220^{low}$ cells from $PU.1^{-/loxP}$ knockout mice) as well as Mac-1^+ macrophages (Mϕ) from both groups and amplified with the primer combination 1/2/3. Primer combination 1/2 shows amplification of the WT (+) and floxed PU.1 alleles (LoxP) as different sized bands; primers 1/3 show the deleted allele (−). Detection of Crewas used as a control for DNA input. (C) FACS analysis of bone marrow cells from a control and a conditional knockout mouse. $CD19^+IgM^-$ cells, corresponding to pro/pre–B cells, and $CD19^+IgM^+$ cells, corresponding to immature cells were gated and the proportion of $EYFP^+$ cells (representing the fraction of cells with active Cre recombinase) shown below. The numbers indicate percentage of cells in the corresponding quadrants. (D) Histogram summarizing the results obtained with pro/pre–B and immature B cells from bone marrow and with mature B-2 cells ($B220^+CD19^+IgD^+$) from spleen and blood. Error bars represent means ± standard deviations. The numbers of mice analyzed are indicated.

disturbed and $B220^{low}EYFP^+$ cells were dispersed throughout the whole spleen (Fig. 6 D). Additional criteria to distinguish B-2 cells from B-1 cells are IgD/IgM expression and size. As shown in Fig. 6 (E and F), $CD19^+$ B cells from the 14-mo-old knockout exhibited high IgM and low IgD expression and were larger in size than control B-2 cells, thus resembling B-1 cells. Whereas the splenic B cell architecture was disrupted, the total $CD19^+$ cell number, although highly variable between mice, showed no notable difference between the two groups (Fig. 6 G). Together, our results indicate that the in vivo deletion of PU.1 in the B cell lineage induced a gradual shift of B-2 cells toward B-1–like cells, which eventually led to an altered structure of the spleen.

B-1 cells replace B-2 cells in the peritoneum of PU.1 conditional knockout mice

Another dramatic change in the distribution of cells within the B cell subsets was observed within the peritoneal cavity. As shown in Fig. 7 A, even fairly young (6 mo old) conditional PU.1 knockout mice contained essentially only B-1

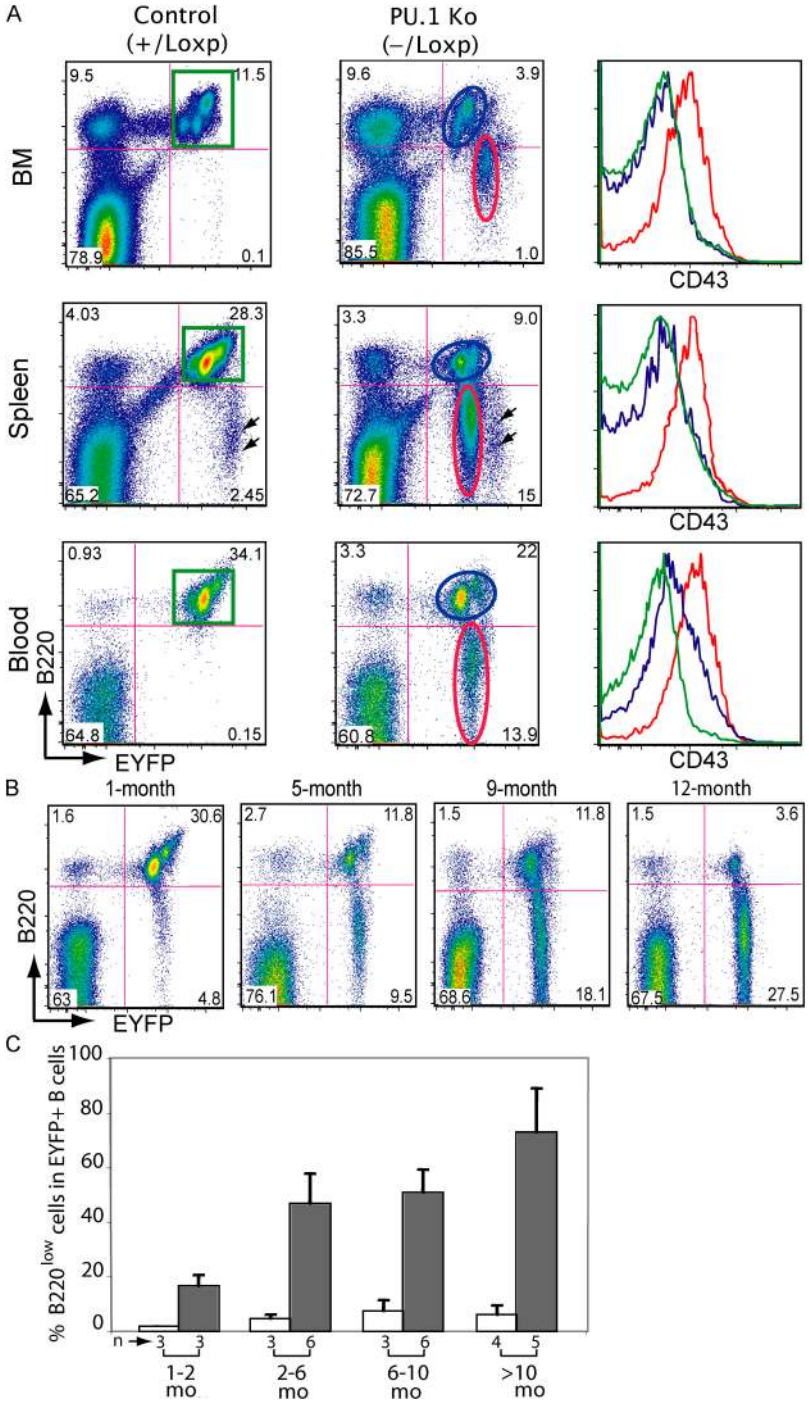

Figure 5. B cells with an altered phenotype can be detected in conditional PU.1 knockout mice and increase with the age. (A) FACS plots of the bone marrow, spleen, and blood leukocytes in a control and a conditional knockout mouse at 6 mo old. The plots show B220 versus EYFP expression. Arrows indicate the EYFP^{bright} plasma cells in the control and the knockout spleen. Histograms show levels of CD43 expression of EYFP^{+} cells within the gates indicated in green for B220^{+} control cells; in blue for B220^{+} knockout cells; and in red for B220^{low} knockout cells. (B) FACS plots of the peripheral blood of PU.1 knockout mouse 25 at 1, 5, 9, and 12 mo old. (C) Histogram summarizing the proportion of B220^{low}EYFP^{+} cells in EYFP^{+} B cell population within the peripheral blood in control (white bars) and knockout mice (black bars) at different age. Error bars represent means ± standard deviations. The numbers of mice analyzed are indicated.

cells (B220$^{neg/low}$) but no B-2 cells (B220^{high}), whereas control mice contained similar proportions of B-1 and B-2 cells. To eliminate the possibility that the PU.1$^{-/-}$ B-1 cells observed represent B-2 cells that simply have down-regulated B220, we measured their forward and sideward scatter properties as well as their IgM/IgD expression. As shown in

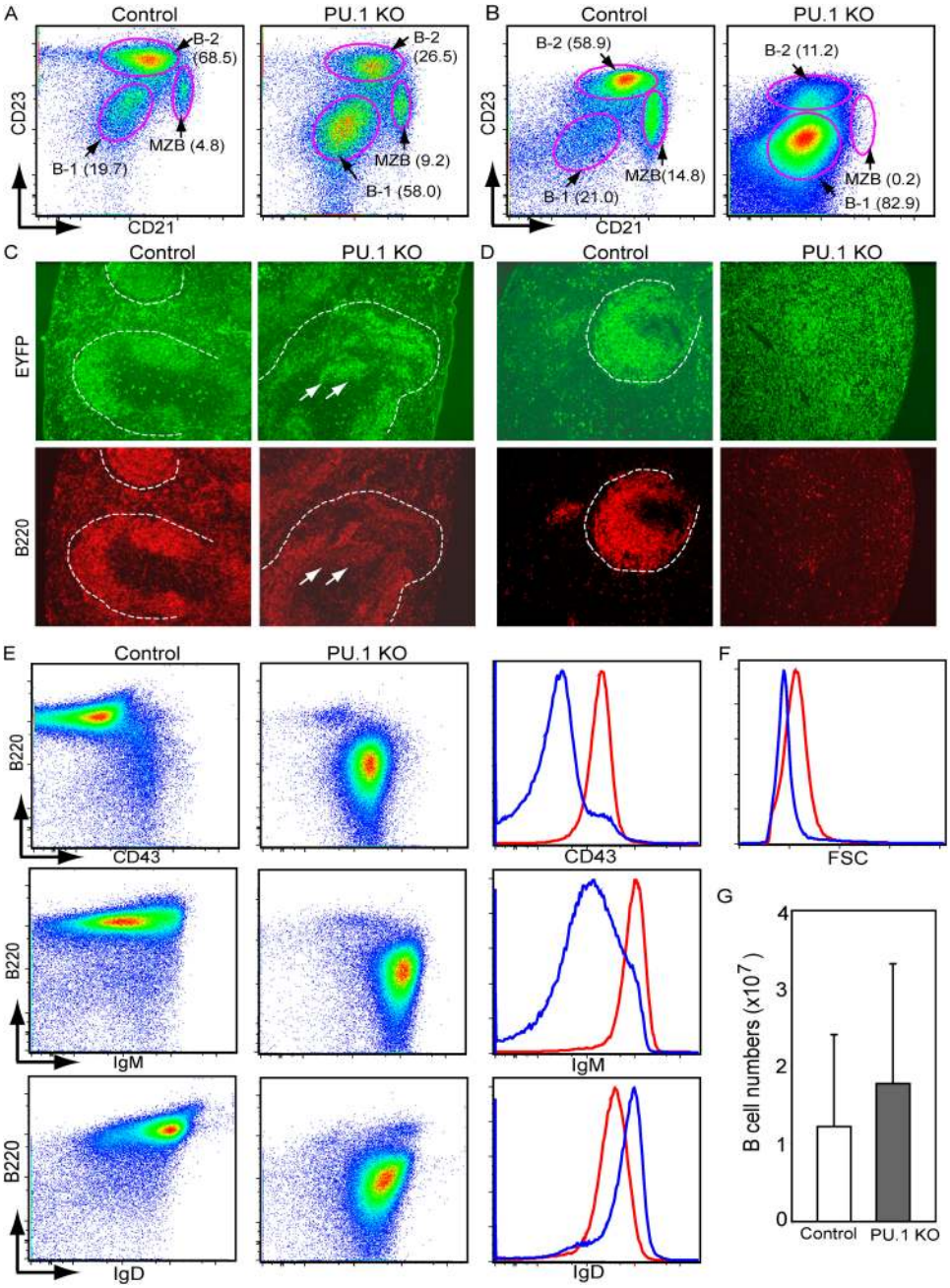

Figure 6. Analysis of B cell subsets in the spleen of 6- and 14-mo-old mice. (A and B) Distribution of B-1, B-2, and MZB cells in 6-mo and 14-mo-old control and knockout littermates, with numbers indicating the percentages in the corresponding gates (pink circles). The splenocytes were isolated and analyzed by FACS, after gating on CD19 positive cells. (C and D) Immunofluorescence analysis of splenic cryosections from the same mice analyzed in A and B. Sections were stained with PE-coupled B220. EYFP expression tracks B cells in which Cre has excised both PU.1 and the stop cassette of the reporter. The primary follicles are circled with dashed white lines, and EYFP$^+$B220^{low} cells in the knockout are indicated by arrows. Original magnification, 10. (E) FACS plots of the CD19$^+$ splenocytes from the 14-mo-old control and knockout littermates. The plots show B220 versus CD43, IgM, and IgD expression, respectively; the histograms show levels of CD43, IgM, and IgD expression in CD19$^+$ cells from the control (blue) and the knockout (red). (F) Histograms of forward (FSC) scatter distribution of CD19$^+$ cells from the control (blue) and the knockout (red). (G) Absolute number of CD19$^+$ B cells in spleen of control (white bar, $n = 7$, 6–9 mo old) and knockout mice (black bar, $n = 10$, 6–9 mo old). Error bars represent means ± standard deviations.

Fig. 7 (B and C), they resembled control B-1 cells in size, granularity, and IgM expression. However, they expressed lower levels of IgD and were negative for Mac-1 and CD5 expression (not depicted). In spite of the virtual disappearance of the B-2 cell compartment in the peritoneum of the conditional knockouts (Fig. 7 D), the numbers of CD19$^+$

cells were comparable with those of WT mice (Fig. 7 E). It is not likely that this was caused by the death of B-2 cells, because we did not see an increase of dead cells in CD19$^+$ population in spleen peritoneum, as measured by DAPI staining. To investigate the possibility that the B-2/B-1 imbalance was caused by an enhanced proliferation of B-1 cells, we sorted B-1 and B-2 cells from WT mice, as well as B-1–like cells from knockouts and placed them in culture (without stroma or added cytokines) for 2 d. A thymidine incorporation assay that cultured the cells for 2 d and labeled for 12 h showed no notable differences between the knockout and control cells (Fig. 7 F). We also measured proliferation responses of the cells to LPS. As shown in Fig. 7 G, the thymidine incorporation values of knockout B-1–like cells (and to a lesser extent of control B-1 cells) were increased after LPS treatment, whereas those of B-2 cells isolated from the peritoneal cavity remained unchanged. In summary, conditional inactivation of PU.1 causes an increase of cells resembling B-1b cells in the peritoneum at the expense of B-2 cells. Short-term ex vivo culture assays suggest that this imbalance was not the result of an increased proliferation capacity of B-1 cells lacking PU.1.

Deletion of PU.1 in B cell precursors induces the gradual down-regulation of B220 expression

The observed B-2/B-1 cell imbalance in conditional PU.1 knockout mice suggested the possibility that the deletion of PU.1 in B2 cells causes them to become B-1–like cells. This implies that B-1 cells require less PU.1 for their formation and maintenance. To test this possibility, we performed a semi-quantitative RT-PCR analysis of peritoneal B-1 and B-2 cells and the PCR products were quantified. As shown in Fig. 8 A, B-2 cells from a WT animal have about eight-fold higher levels of PU.1 than B-1 cells, although B-1 cells were not negative. To directly test whether B-2 cells can be induced to switch to become B-1 cells when PU.1 is inactivated, we isolated CD19$^+$ cells from the bone marrow of PU.1$^{+/loxP}$ROSA26-EYFP$^{+/-}$ mice and placed them in culture under conditions supporting pro/pre–B cell development (S17 stroma plus cytokines). The cells were then incubated with nuclear localized Cre recombinase protein fused to TAT (20). Induction of EYFP expression in these cultures indicated cells in which PU.1 became deleted. When the cultures were analyzed 9, 14, and 40 d later, the EYFP$^+$ cells were found to gradually down-regulate B220 expression, a process resembling the slow reprogramming of B-2 cells in vivo. In addition, there was a selective increase in the proportion of EYFP$^+$ cells without appreciable induction of cell death, suggesting that the PU.1 knockout cells proliferate more rapidly under these culture conditions (Fig. 8 B). CD43 could not be used as an additional marker to monitor phenotypic changes because the culture conditions used favor the growth of CD43$^+$ pro–B cells. Together, these observations are consistent with the possibility that deletion of PU.1 induces the reprogramming of B cell progenitors into B-1–like cells.

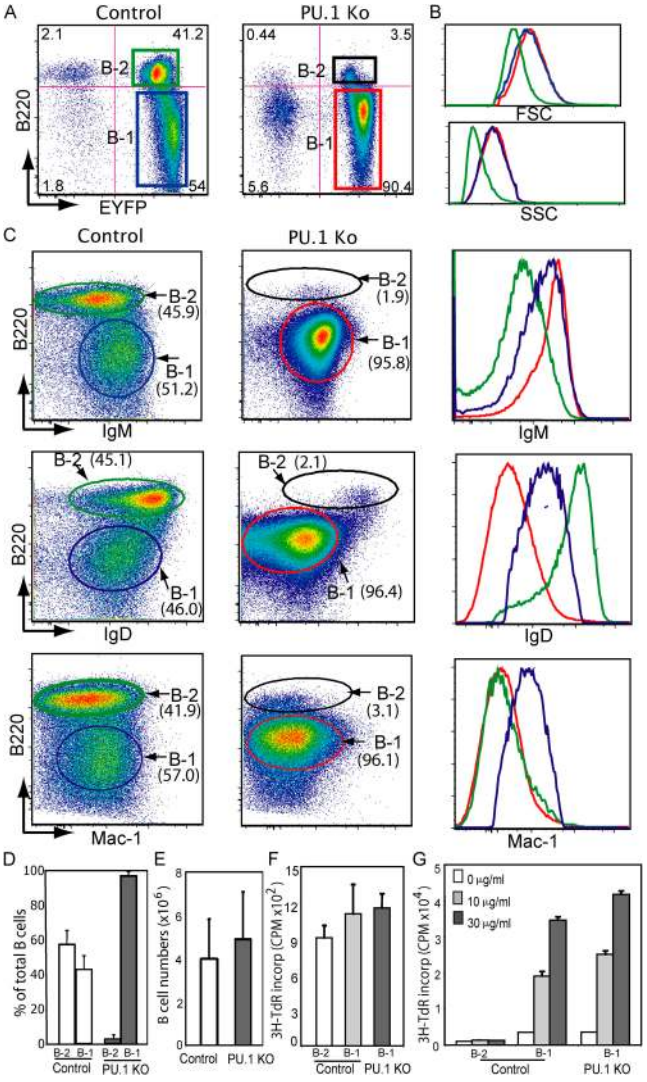

Figure 7. Enrichment of B-1–like cells in the peritoneal cavity of conditional knockout mice. (A) FACS plots of CD19$^+$ peritoneal cells in 6-mo-old control and knockout littermates, showing B-2 and B-1 cells and percentage of cells present in each quadrant. (B) Forward (FSC) and side (SSC) scatter distribution of cells within the gates indicated in the plots (green, control B-2 cells; blue, control B-1 cells; red, knockout B-1 cells). (C) B220 expression versus IgM, IgD, and Mac-1 expression, respectively, in CD19$^+$ peritoneal cells. The B-1 and B-2 subsets are indicated, with number in parentheses representing percentage of cells in the corresponding gate (circles). Histograms show levels of IgM, IgD, and Mac-1 expression of cells within the gates indicated in the plots. (D) Histogram summarizing the proportion of B-1 and B-2 cells within the peritoneal B cell population in control (white bars, $n = 4$, 6 mo old) and knockout mice (black bars, $n = 5$, 6 mo old) with mean values and standard deviation. (E) Absolute number of CD19$^+$ B cells in the peritoneal cavity of controls (white bar, $n = 4$, 6 mo old) and knockouts (black bar, $n = 5$, 6 mo old). Error bars represent means ± standard deviations. (F) The proliferation capacity of peritoneal B subsets in vitro without stimulation. B cell subsets were sorted from the peritoneal wash from two 6-mo-old control mice and knockouts and placed in culture for 2 d. DNA synthesis was quantified by measuring [^{3}H]thymidine incorporation. (G) Effect of the stimulation of LPS on the proliferation capacity of peritoneal B cells in vitro. (F and G) Control B-2 cells, control B-1 cells, and knockout B-1 cells are represented by white, gray, and black bars, respectively. Data are representative of two experiments performed in triplicate.

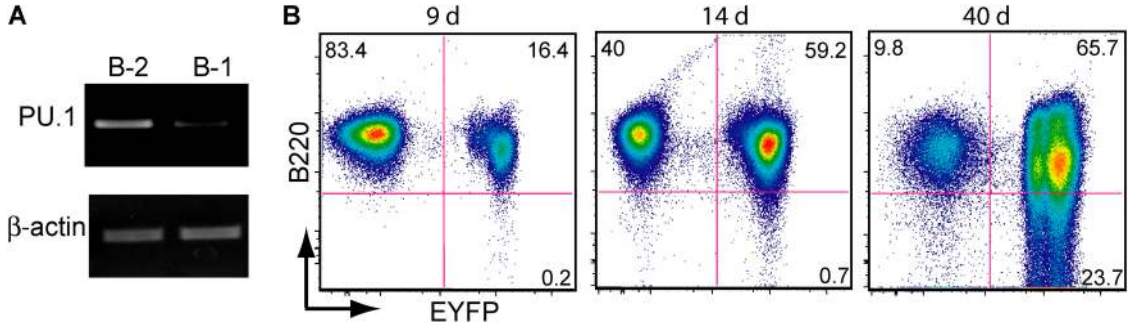

Figure 8. PU.1 expression in peritoneal B cells and the effect of PU.1 ablation. (A) RT-PCR analysis of PU.1 expression in sorted WT B-2 ($B220^{hi}CD19^{+}IgM^{lo}$) and B-1 cells ($B220^{low/neg}CD19^{+}IgM^{hi}$). (B) Effect of PU.1 ablation on the phenotype and growth of B progenitor cells in vitro. $CD19^{+}$ cells were isolated from the bone marrow of a $PU.1^{-/loxP} \times$ ROSA26-$EYFP^{+/ki}$ mouse, treated with soluble Cre-TAT protein, and were seeded on S17 stroma. The FACS plots show cells analyzed 9, 14, and 40 d later for the expression of B220 and EYFP. The numbers indicate the percentages of cells in the corresponding quadrants.

DISCUSSION

Lineage specification and commitment in the absence of PU.1: implications for models of B cell development

Contrary to what was expected from the literature, we found that B lineage cells can develop in the absence of PU.1 when knockout fetal liver cell suspensions are placed in culture. The cells express all early B cell genes tested (except CD45/B220), contain immunoglobulin gene rearrangements, and can produce IgM. Previous articles had described that no B lineage cells can be grown from $PU.1^{-/-}$ fetal liver, unless IL-7R or EBF was expressed in these cells (5, 7). This apparent discrepancy does not reflect differences between different PU.1 mutant alleles since our observations were reproduced with a well-characterized knockout line (Singh, H., personal communication). The explanation therefore is that because of the extended latency of outgrowth of $PU.1^{-/-}$ B cells compared with WT cells, exceeding the times at which such cultures are traditionally kept, they had previously been overlooked. We do not think that the growth of the PU.1-defective cells is caused by spontaneous mutations that activate the expression of the IL-7R or EBF genes. Thus, the frequency of outgrowths observed ($PU.1^{-/-}$ B cells were obtained from all 21 $PU.1^{-/-}$ fetal livers tested within a time frame of 2–3 wk, with >5 colonies per fetal liver) cannot be explained by the known mammalian mutation rates of 10^{-7}–10^{-9} (for review see reference 21).

Our in vitro experiments indicate that the transcription factor network that establishes and maintains B cell fate has a built in robustness that permits it to assemble and perform even in the absence of a major player. At the same time they suggest that PU.1 facilitates commitment by rapidly activating downstream target genes such as EBF and IL-7R. A somewhat similar situation applies to T cell development: here PU.1 is also not strictly required but acts as a facilitator, as T cells develop at low frequencies and delayed appearance in $PU.1^{-/-}$ mice in vivo and in vitro (2, 22, 23). That PU.1-defective B cells do not inappropriately express erythro/myeloid cell restricted genes and cannot be induced to differentiate into macrophages in the absence of IL-7 differs from findings with B lineage cells lacking other transcription factors required for B cell development. Pro–B cells that are defective for either Pax5 or E2A contain IgH DJ rearrangements, yet inappropriately express myeloid- and T cell–restricted genes and can be induced to differentiate into several myeloid lineages, as well as T cells and NK cells (24, 25). However, although $PU.1^{-/-}$ B cells do not become T cells on OP9-DL1 stroma cells (reference 26; unpublished data), it is striking that they express *lck* and *ZAP-70*, which are genes that are normally expressed in normal T and NK cells. In addition, ZAP-70 is expressed in an aggressive subset of B cell chronic lymphocytic leukemia (27). It will be interesting to determine whether these leukemic cells lack PU.1 expression; and if so, whether the ablation of PU.1 is a causative factor in their formation.

PU.1 as a transcriptional regulator of B cell genes

The observation that B lineage cells express IL-7R and EBF genes in the absence of PU.1 implies that the factor is dispensable for their regulation. It is not likely that this is because of a redundancy with the closely related transcription factor Spi-B since the outgrowth of $PU.1^{-/-}$B lineage cells was observed even when Spi-B was absent (Singh, H., personal communication). Similarly, IL-7R has been shown to induce the outgrowth of B cells from $PU.1^{-/-}$Spi-$B^{-/-}$ fetal liver (12). Therefore, although PU.1 is not required for the expression of the IL-7R and EBF genes, it probably enhances their initial up-regulation in collaboration with other transcription factors, such as E2A (28). A different mechanism applies to the regulation of the PU.1 target gene CD45 (29). Although this gene is not expressed in fetal liver-derived $PU.1^{-/-}$ B cells, it is expressed in $B220^{low}EYFP^{+}$ B cells from PU.1 conditional knockout mice (unpublished data). This suggests that the de novo expression of CD45, but not its maintenance, requires PU.1; this is a situation that is reminiscent of T cells, which express CD45 even after they have down-regulated PU.1 (30). It also raises the possi-

bility that PU.1 is involved in the regulation of B220 expression at more than one level. B220 corresponds to a glycosylated full-length CD45 molecule, which contains a carbohydrate epitope recognized by the RA3-6B2 antibody (the clone used for B220 detection) (31, 32). Thus, the observed difference between CD45 and B220 expression in $EYFP^+$ B cells from PU.1 knockouts raises the possibility that PU.1 might participate the regulation of a B cell–specific glycosylation enzyme.

B-2 to B-1 cell conversion mediated by PU.1 loss

The most striking effect of deleting PU.1 within the B cell lineage is a decrease in B-2 cells and an increase in B-1 cells. In principle, this shift can either be explained by an enhanced proliferation of B-1 cells, the death of B-2 cells, or an induced reprogramming of B-2 cells into B-1 cells. Several arguments suggest that the latter possibility is the predominant mechanism. Peritoneal B-1–like cells from knockout mice showed a similar proliferation capacity as the controls, and there was no net increase in the total number of B cells in the conditional knockout animals. (The observed increased in vitro proliferation of B cell progenitors after PU.1 ablation might be caused by the culture conditions that mimic the bone marrow environment. We speculate that, in vivo, these cells leave the bone marrow and relocate in the spleen and peritoneum, where they do not appear to proliferate.) We also did not observe substantial cell death induced by PU.1 deletion, supporting the finding that excision of PU.1 in CLPs does not induce a reduction of their colony forming efficiency (33). Finally, excision of PU.1 in ex vivo cultures of B cell precursors induced a slow downregulation of B220, reflecting the gradual changes observed in vivo. These $B220^{neg/low}$ cells most likely do not originate from B-1 cell precursors present in the bone marrow because a decrease of B220 expression could be detected in the bulk of the $EYFP^+$ population already after 9 d, although selection cannot be ruled out completely. Another argument for a B-2 to B-1 cell conversion is the finding that the level of B220 expression in the B-1–like population is highest in the bone marrow, intermediate in the spleen, and lowest in the peritoneum. This supports the idea that reprogramming is initiated in bone marrow B cell precursors and completed after the cells migrate to the spleen and peritoneum.

The observed complete loss of B-2 as well as of MZB cells in old mice indicates that deletion of PU.1 also induces mature B-2 cells to acquire a B-1–like phenotype. In 6-mo-old conditional knockouts, although the $EYFP^+B220^+$ follicular B cells are essentially unchanged, they express slightly higher levels of CD43 and are slightly larger than their normal counterparts (Fig. 5 A and not depicted). Similarly, at this stage MZB cells, which are thought to originate from mature B-2 cells (12), exhibit a decrease in the expression of CD21, possibly because they are in an early phase of the reprogramming process. In comparison with the spleen, the shift from B-2 to B-1 cells in the peritoneum appears to occur much earlier and faster (such a shift was already seen in a 2-mo-old knockout mouse, before changes in the spleen were apparent). Perhaps the peritoneal environment facilitates the conversion of B-2 to B-1 cells.

Our proposal that the loss of PU.1 induces a B-2 to B-1 cell switch is based on the assumption that the resulting B-1 cells correspond to normal B-1 cells. Indeed, peritoneal knockout B-1–like cells are indistinguishable from WT B-1b cells in that they are $B220^{neg/low}CD21^{low}CD23^{low}CD43^{med}IgM^{high}$, large in size and highly granular. However, they differ in the expression of IgD, Mac-1, and enhanced proliferation response to LPS. Together with the finding that normal B-1 cells express much lower levels of PU.1 than B-2 cells, this suggests that B-1 cells represent, in part, a PU.1 loss of function phenotype. The finding that the B-1–like cells in the knockout mice are all CD5 negative might be explained by the possibility that low levels of PU.1 drives CD5 expression in the B-1a subset.

Our observations support the notion that the level of PU.1 is a decisive factor in the decision between B-1 and B-2 cell fates. To further test this hypothesis, it will be necessary to determine whether overexpression of PU.1 induces B-1 cells to acquire a B-2 cell phenotype. The observed increase in the ratio of B-1 to B-2 cells resembles that seen in transgenic animals engineered to exhibit high levels of BCR signaling (15). It will therefore be interesting to know whether strong BCR signaling leads to inhibition of PU.1 expression or activity.

MATERIALS AND METHODS

Mice. Germline-deleted $PU.1^{+/-}$ mice were obtained after crossing β-actin–Cre mice (reference 34; mice provided by D. Tenen, Harvard Institute of Medicine, Boston, MA) with a line in which exons 4 and 5 are flanked by loxP sequences ($PU.1^{+/loxP}$) (33). Progeny exhibiting a germline deletion of the stop cassette were crossed with C57BL mice to remove the β-actin–Cre transgene. The $PU.1^{+/-}$ mice were then crossed with each other to obtain litters containing homozygous knockout embryos. The strategy used to generate mice in which PU.1 is selectively inactivated in B cells (conditional knockouts), and in which the cells with the deletion can be tracked, is shown in Fig. S2. In short, $PU.1^{loxP/loxP}$ mice were crossed with $CD19\text{-}Cre^{ki/ki}$ $ROSA26\text{-}EYFP^{ki/ki}$ $PU.1^{+/-}$ mice. This generated $CD19\text{-}Cre^{+/ki}$ $ROSA26\text{-}EYFP^{+/ki}$ $PU.1^{+/loxP}$ (conditional knockout) and $CD19\text{-}Cre^{+/ki}$ $ROSA26\text{-}EYFP^{+/ki}$ $PU.1^{-/loxP}$ (control) mice. The genetic background of the PU.1 germline and conditional knockout lines is a mixture of C57Bl6 and 129 lines. The origin of the $CD19\text{-}Cre^{ki}$ and $ROSA26\text{-}EYFP^{ki}$ mice was described earlier (35). Mice were maintained in the Research Animal Facility at Albert Einstein College of Medicine in accordance with guidelines from the Institute for Animal Studies.

Mouse genotyping. $PU.1^{+/-}$ and $PU.1^{+/loxP}$ mice were genotyped by PCR analysis of tail DNA with the following primers: 5′-CTT CAC TGC CCA TTC ATT GGC TCA TCA-3′ (PU.1 primer 1); 5′-GCT GGG GAC AAG GTT TGA TAA GGG AA-3′ (PU.1 primer 2); and 5′-GCT TGT GGA GTC TCC ATT AAG AAT GC-3′ (PU.1 primer 3). PCR fragments of 400- and 500-bp products of the combination of primers 1 and 2 were indicative of WT and floxed alleles, respectively. The deletion gave rise to PCR products of 400 bp using primers 1 and 3. The oligonucleotides used for PCR genotyping of CD19-Cre mouse and ROSA26 reporter mouse were generated as described previously (35). The β-actin–Cre transgenic mice were genotyped with the Cre-specific primers 5′-CAATT-TACTGACCGTACAC-3′ and 5′-TAATCGCCATCTTCCAGCAG-3′,

which resulted in a 1-kb PCR product. All reactions were performed with 100 ng DNA for 30 cycles of 30-s denaturation at 94°C, 30-s annealing at 60°C, and 60-s elongation at 72°C. The products were analyzed on a 1.5% agarose gel.

Growth of fetal liver–derived B lineage cells. Fetal liver cells were cultured on S17 stromal cells in the presence of 5 ng/ml IL-7, 10 ng/ml SCF, and 10 ng/ml Flt-3 ligand in MEM α medium (GIBCO BRL) plus 10% FCS (Gemini Bio Products). WT B220-positive pre–B cells were isolated to >95% purity from fetal liver by flow sorting and cultured as above.

PCR analysis of heavy chain DJ and VDJ rearrangements. PCR conditions and primers have been described in detail elsewhere (17, 36). Amplified DNA products were analyzed on a 1.5% agarose gel and was followed by ethidium bromide staining. PCR products were excised from the gel, cloned, several clones were sequenced, and the reading frames of individual sequences were determined.

Limiting dilution analysis of B cell precursors. E16.5 fetal liver cells from two PU.1$^{-/-}$ embryos were pooled as well as cells from one PU.1$^{+/-}$ together with two PU.1$^{+/+}$ embryos. LSK cells were sorted from the two samples and 2, 5, 10, 20, 40, 80, 160, 320, 640, and 1,280 cells/well plated in six wells each of a 96 well plate containing irradiated S17 stroma plus cytokines IL-7, SCF, and Flt-3 ligand. The number of B cell colonies was scored under the microscope between 4 and 22 d of culture. The B cell identity of the cells in the colonies was confirmed by FACS analysis for a limited number of the wells.

Flow cytometry and cell sorting. 10^6 cells were washed once in PBS and resuspended in 100 μl of FACS buffer (4% FCS in PBS plus 0.01% sodium azide) containing 0.5 μg of antibody. Cells were stained in the dark on ice for 30 min. After washing, the cells were resuspended in 1 ml of FACS buffer and analyzed by flow cytometry. CD45, B220, CD43, CD19, BP-1, CD24, IgM, IgD, CD21, CD23, Mac-1, Gr-1, CD3, CD4, CD8, NK1.1, and TER119 antibodies were purchased from BD Biosciences. Viable cells were identified by DAPI exclusion. Cells were analyzed using FACScalibur and LSR-II flow cytometers (Becton Dickinson) and sorted with a MoFlo cell sorter (DakoCytomation).

RT-PCR analysis. Total RNA was isolated and converted into cDNA as described previously (36). All RT-PCR primers have been published (36) except the following: PU.1 sense, 5′-CCTACATGCCCCGGATGTGC-3′ and antisense, 5′-TGCTGTCCTTCATGTCGCCG-3′; GATA-1 sense, 5′-GACCCCCCCTTTTTTTGGAG-3′ and antisense, 5′-GAGGCACAAGGATCTGAAGCT-3′; pre-Tα sense, 5′-CATGCTTCTCCACGAGTG-3′ and antisense, 5′-CTATGTCCAAATTCTGTGGGTG-3′; lck sense, 5′-CAGAGGGCATGGCGTTCAT-3′ and antisense, 5′-CTGTGGCTGTGAAGAAGTC-3′ and ZAP-70 sense, 5′-GGAGAAGAACTTTGTGCACCG-3′ and antisense, 5′-ATGGTGACCCCATAGCTCCA-3′. ImageQuant software (Molecular Dynamic) was used for the quantitation of PCR products.

Ablation of PU.1 with soluble TAT-Cre. CD19$^+$ cells were sorted from the bone marrow of a PU.1$^{-/loxP}$ ROSA26-EYFP$^{+/ki}$ mouse and treated with 0.5 μM TAT-Cre (provided by S. Dowdy, Howard Hughes Medical Institute, University of California, San Diego, CA) for 1 h in growth medium without FCS. Cells were then washed once with medium and plated on S17 stroma cells in the presence of cytokines, IL-7, SCF, and Flit-3L. Several days later, the cells were analyzed for successful excision of the stop cassette of the reporter gene by monitoring EYFP expression.

B cell proliferation assay. Peritoneal cells were obtained by flushing the peritoneal cavity with RPMI 1640 supplemented with 10% FCS and 1 mM EDTA. B cells were isolated in a two-step process. First, macrophages were depleted by panning the adherent cells for 2 h at 37°C. This was followed by FACS sorting. The B-2 cells from control mice were identified as EYFP$^+$B220^{hi}Mac-1$^-$ and the B-1 population was EYFP$^+$B220^{low}Mac-1^{low}, whereas B-1 cells from the knockouts were EYFP$^+$B220$^{low/-}$. Sorted cells (5×10^5 cells/well) were cultured in RPMI 1640 containing 10% FCS and 10^{-4} M β–mercaptoethanol in a 96-well plate without or with LPS (10 and 30 μg/ml; Sigma-Aldrich). After 2 d of incubating, cells were labeled with 1 μCi [^{3}H]TdR per well (Amersham Biosciences). 8 h later, [^{3}H]TdR incorporation was measured using a β scintillation counter (PerkinElmer). All measurements were in triplicate and the assay was repeated twice.

Online supplemental material. Two supplemental figures are provided. Fig. S1 shows the analysis of freshly isolated E16.5 fetal liver cells from WT (+/+), PU.1 heterozygous (+/−), and PU.1 knockout (−/−) embryos. Fig. S2 presents a scheme that describes the breeding process by which the B cell–specific PU.1 knockout mouse model was developed. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20051089/DC1.

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