

# Perturbed myelo/erythropoiesis in *Lyn*-deficient mice is similar to that in mice lacking the inhibitory phosphatases *SHP-1* and *SHIP-1*

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**The *Lyn* tyrosine kinase plays essential inhibitory signaling roles within hematopoietic cells by recruiting inhibitory phosphatases such as SH2-domain containing phosphatase-1 (SHP-1), SHP-2, and SH2-domain containing 5'-inositol phosphatase (SHIP-1) to the plasma membrane in response to specific stimuli. *Lyn*-deficient mice display a collection of hematopoietic defects, including autoimmune disease as a result of autoantibody production, and perturbations in myelopoiesis that**

**ultimately lead to splenomegaly and myeloid neoplasia. In this study, we demonstrate that loss of *Lyn* results in a stem/progenitor cell-intrinsic defect leading to an age-dependent increase in myeloid, erythroid, and primitive hematopoietic progenitor numbers that is independent of autoimmune disease. Despite possessing increased numbers of erythroid progenitors, and a more robust expansion of these cells following phenylhydrazine challenge, *Lyn*-deficient mice are more**

**severely affected by the chemotherapeutic drug 5-fluorouracil, revealing a greater proportion of cycling progenitors. We also show that mice lacking SHIP-1 have defects in the erythroid and myeloid compartments similar to those in mice lacking *Lyn* or SHP-1, suggesting an intimate relationship between *Lyn*, SHP-1, and SHIP-1 in regulating hematopoiesis. (Blood. 2004;104:3901-3910)**

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## Introduction

The production and lineage commitment of hematopoietic cells is governed by the actions of a multitude of cytokines, hormones, and growth factors that bind to cell surface receptors activating signal transduction cascades that ultimately regulate the expression of genes that control cell fate and effector function.<sup>1</sup> Signal propagation in these cells is actively counterbalanced by several families of inhibitory gene products including protein tyrosine phosphatases,<sup>2</sup> phosphatidylinositol phosphatases,<sup>3</sup> the suppressors of cytokine signaling (SOCS) proteins,<sup>4</sup> and receptors bearing immunoreceptor tyrosine-based inhibitory motifs (ITIMs).<sup>5</sup> The central role played by tyrosine phosphorylation is exemplified by mutations in particular genes that lead to deregulation of hematopoiesis. For example, mutational activation of either the *Abl*<sup>6,7</sup> or Janus tyrosine kinases<sup>8-10</sup> leads to leukemogenesis. Loss of appropriate negative regulation of signaling may also have catastrophic consequences. For example, loss-of-function mutations within the inhibitory phosphatase Src homology 2 (SH2)-containing phosphatase-1 (*SHP-1*)<sup>11-13</sup> in *motheaten* and *motheaten viable* mice (*Me<sup>v</sup>*), or disruption of the murine SH2-domain containing 5'-inositol phosphatase (*SHIP-1*)<sup>14,15</sup> gene, lead to severe perturbations in hematopoiesis with myeloid cell consolidation of the lungs of deficient mice leading to premature death.<sup>14-16</sup> Thus, the appropriate balance of positive and negative elements of signal transduction is essential for maintaining normal hematopoietic cell self-renewal, differentiation, and immune cell function.

Although clearly involved in initiating tyrosine-phosphorylation cascades following hematopoietic cell stimulation,<sup>17</sup> *Lyn* has emerged as a critical enzyme responsible for establishing signaling thresholds in B cells,<sup>18-21</sup> myelomonocytic cells,<sup>22,23</sup> and mast cells.<sup>24-28</sup> Indeed, loss of *Lyn* kinase leads to defects in activation of inhibitory phosphatases that likely underlies the hypersensitivity of deficient cells to immunoreceptor and cytokine stimulation.<sup>20,22,29,30</sup> In the case of B cells and mast cells, *Lyn* deficiency is associated with impaired activation of spleen tyrosine kinase (Syk), but also with enhanced immunoreceptor-dependent activation of AKT and mitogen-activated protein (MAP) kinases, as well as heightened growth factor or immunoreceptor-dependent proliferation.<sup>18,19,24-27,31</sup> In macrophages, *Lyn* deficiency leads to enhanced sensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage-CSF (M-CSF), diminished phosphorylation of SHIP-1 and SHP-1, enhanced AKT activity, and improved survival in cytokine-reduced conditions.<sup>22,23</sup> Similarly, loss of either SHIP-1 or SHP-1 leads to enhanced hypersensitivity to growth factors and resistance to cytokine withdrawal-induced cell death.<sup>14,15,32,33</sup>

We have previously shown that *Lyn*<sup>-/-</sup> mice exhibit hematopoietic system defects that lead to an age-dependent increase in extramedullary myelopoiesis and widely disseminated myeloid neoplasia. In this study, we have characterized the temporal development of perturbed hematopoiesis, identified an increase in erythroid progenitor numbers in these mice, and conducted a

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comparative analysis of the *Lyn*<sup>-/-</sup> phenotype with both *Me<sup>v</sup>/Me<sup>v</sup>* and *SHIP-1*<sup>-/-</sup> mice. We show that *Lyn*<sup>-/-</sup> mice develop a similar, although much less severe, defect in hematopoiesis than *Me<sup>v</sup>/Me<sup>v</sup>* and *SHIP-1*<sup>-/-</sup> mice, which is characterized by dramatically increased splenic progenitors and progeny of the myeloid and erythroid lineages. Furthermore, we show that these characteristics of *Lyn*-deficient mice are transplantable with bone marrow (BM) and independent of B cells and autoimmune disease.

## Materials and methods

### Mice

*Lyn*<sup>-/-34</sup> (mixed 12901a × C57BL/6 intercross or C57BL/6 generation 20 background), *SHIP-1*<sup>-/-15</sup> (mixed 129 × C57BL/6 intercross), *Me<sup>v</sup>/Me<sup>v</sup>* (C57BL/6), *μMT/μMT*<sup>35</sup> (C57BL/6), and *Lyn*<sup>-/-;μMT/μMT</sup> (mixed 12901a × C57BL/6) mice were housed in microisolation units. Animal experimentation was performed in accordance with National Health and Medical Research Council of Australia (NHMRC) guidelines.

### Progenitor and colony-forming unit–spleen 12 (CFU-S<sub>12</sub>) assays

Myeloid colony-forming numbers were determined in semisolid agar.<sup>22</sup> Erythroid progenitors assays were conducted in methylcellulose (Methocult; StemCell Technologies, Vancouver, BC) in 4 units/mL erythropoietin (EPO, Eprex 1000; Janssen-Cilag, North Ryde, Australia) alone (colony-forming unit–erythroid [CFU-e]) or EPO, stem cell factor (SCF, 50 ng/mL), and interleukin-3 (IL-3, 10 ng/mL) for burst-forming unit–erythroid (BFU-e). Colonies were scored on day 2 (CFU-e's) or day 10 (BFU-e's) following staining with benzidine dihydrochloride (Sigma, St Louis, MO). Day-12 colony-forming unit–spleen assays were performed by intravenously injecting 75 000 BM or 375 000 spleen cells from matched C57BL/6 *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice into lethally irradiated C57BL/6 recipients (11 Gy). Donor samples were pooled from 3 mice/genotype and injected into 6 recipients. At 12 days after injection, spleens were fixed in Bouin solution, and macroscopic colonies enumerated.

### Treatment with 5-fluorouracil and phenylhydrazine

Matched *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> C57BL/6 background mice (5 mice/time point) were intravenously injected with 150 mg/kg 5-fluorouracil (5-FU). Blood was analyzed on an ADVIA 120 hematology system (Bayer Australia, Pymble, Australia).<sup>22</sup> For phenylhydrazine challenge, mice were intraperitoneally injected on days 1 and 2 with 60 mg/kg per mouse of phenylhydrazine hydrochloride in phosphate-buffered saline (PBS). Reticulocyte numbers (1000 cells/mouse) were determined by staining with New methylene blue (Sigma).

### Transplantation experiments

*B6.SJL-Ptprc<sup>a</sup>* (Ly5.1) congenic mice were intravenously injected with 10<sup>6</sup> BM cells from Ly5.2 C57BL/6 background *Lyn*<sup>+/+</sup> or *Lyn*<sup>-/-</sup> donors following lethal irradiation with 11 Gy of gamma irradiation given in 2 doses 3 hours apart. BM from 3 donor mice was pooled before injection, and 4 to 6 recipients were injected with each donor population/experiment. Irradiated recipient mice were maintained on enrofloxacin (170 mg/L) for 2 weeks. Assessment of engraftment was achieved by fluorescence-activated cell-sorter (FACS) analysis of peripheral blood, BM, and spleen preparations 10 to 12 weeks after transplantation using the Ly5.1/5.2 markers CD45.1/CD45.2, respectively.

### Flow cytometry and ELISA assays

Single-cell suspensions were stained with Ter119 (Ly-76), CD71 (C2), CD45.1 (A20), CD45.2 (104), Mac-1 (M1/70), Gr-1 (Rb6-8C5), or isotype control rat immunoglobulin G2bκ (IgG2bκ, A95-1).<sup>22,36</sup> Assessment of bromodeoxyuridine (BrdU) incorporation into cycling cells was determined according to the manufacturer's instructions (BD Pharmingen, San Diego,

CA). Annexin V–fluorescein isothiocyanate (FITC) staining of fetal liver and BM erythroid cells<sup>37</sup> and measurement of total immunoglobulin<sup>34,36</sup> were carried out as described. Antinuclear antibody (ANA) enzyme-linked immunosorbent assays (ELISAs) were conducted using a commercially available human ANA detection kit (Binding Site, Birmingham, England) adapted to mouse ANA detection.

### Purification of CD71<sup>+</sup> cells

Spleens were harvested from phenylhydrazine-treated mice at day 5 or 6 after injection, homogenized, and then incubated in ice-cold Tris (tris(hydroxymethyl)aminomethane)-buffered NH<sub>4</sub>Cl at 37°C to lyse red blood cells (RBCs). Cell suspensions were then passed through a 40-μm nylon cell strainer after washing in PBS, 0.5% fetal calf serum (FCS), 2 mM EDTA (ethylenediaminetetraacetic acid) (magnetic-activated cell separation [MACS] buffer). Cells were then labeled with 7.5 μL CD71-biotin (C2; BD Pharmingen) and purified using streptavidin microbeads and LS cell separation columns according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD71<sup>+</sup>/Ter119<sup>+</sup> cells was always more than 90% as judged by FACS analysis. Cell yields were routinely between 1.5 and 3 × 10<sup>8</sup> cells/spleen.

### Cell stimulation and lysis

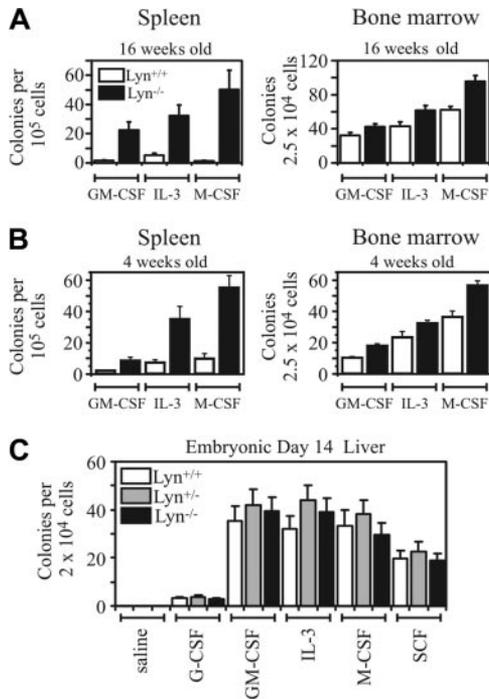
Cells (1.5 × 10<sup>8</sup>) were resuspended in Tyrode solution (124 mM NaCl, 4 mM KCl, 0.64 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.6 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM NaHCO<sub>3</sub>, 5.5 mM glucose, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 5 mM MES [2-(*N*-morpholino)ethanesulfonic acid], 0.1% gelatin; pH 7.65). Samples were preheated at 37°C for 10 minutes before stimulation. Cells were washed in ice-cold Tyrode solution before lysis.<sup>22</sup> Antibodies (Abs) used included anti-PY (4G10; UBI, Lake Placid, NY), anti-EPOr (M-20; Santa Cruz Biotechnology, Santa Cruz, CA), Janus kinase 2 (Jak2, C-20; Santa Cruz Biotechnology), and signal transducer and activator of transcription 5 (STAT5, N-20; Santa Cruz Biotechnology). Phospho-STAT5 and phospho-extracellular signal-related kinase 1/2 (ERK1/2) Abs were from Cell Signaling Technology (Beverly, MA). Rabbit antibodies against SHIP-1 and Lyn were produced by immunizing rabbits with a glutathione-S-transferase (GST) fusion protein containing residues 953 to 1122 of murine SHIP-1 or the SH3/SH2 domains of Lyn (provided by P. Lock, Department of Surgery, University of Melbourne, Australia). Blots were processed as described elsewhere.<sup>22</sup>

## Results

### Age-dependent increase of myeloid progenitors in *Lyn*<sup>-/-</sup> mice

We have previously demonstrated that *Lyn*<sup>-/-</sup> mice develop splenomegaly characterized by a dramatic increase in hematopoietic progenitors responsive to cytokines such as GM-CSF, IL-3, M-CSF, and SCF. Moreover, *Lyn*<sup>-/-</sup> mice develop a myeloproliferative disease characterized by the accumulation of myelomonocytic cells widely disseminated throughout the animals.<sup>22</sup>

In order to understand the temporal progression of this phenotype, we investigated hematopoiesis in embryonic day-14 (E14) fetal liver and 4- and 16-week-old mice. This analysis revealed increased numbers of myeloid progenitors in the spleens of *Lyn*<sup>-/-</sup> mice at both 4 and 16 weeks of age (Figure 1A-B), although, the magnitude of the increase in GM-CSF- and M-CSF-responsive progenitors was enhanced in older mice (GM-CSF/M-CSF progenitors were 13- and 35-fold higher, respectively, in 16-week-old *Lyn*<sup>-/-</sup> mice compared with *Lyn*<sup>+/+</sup> mice, and 4- and 6-fold higher, respectively, in 4-week-old mice). This increase in splenic myeloid progenitors does not appear to be due to an impairment of BM myelopoiesis, as the number of myeloid progenitors in the BM of *Lyn*<sup>-/-</sup> mice was similar to that in wild-type mice at all ages

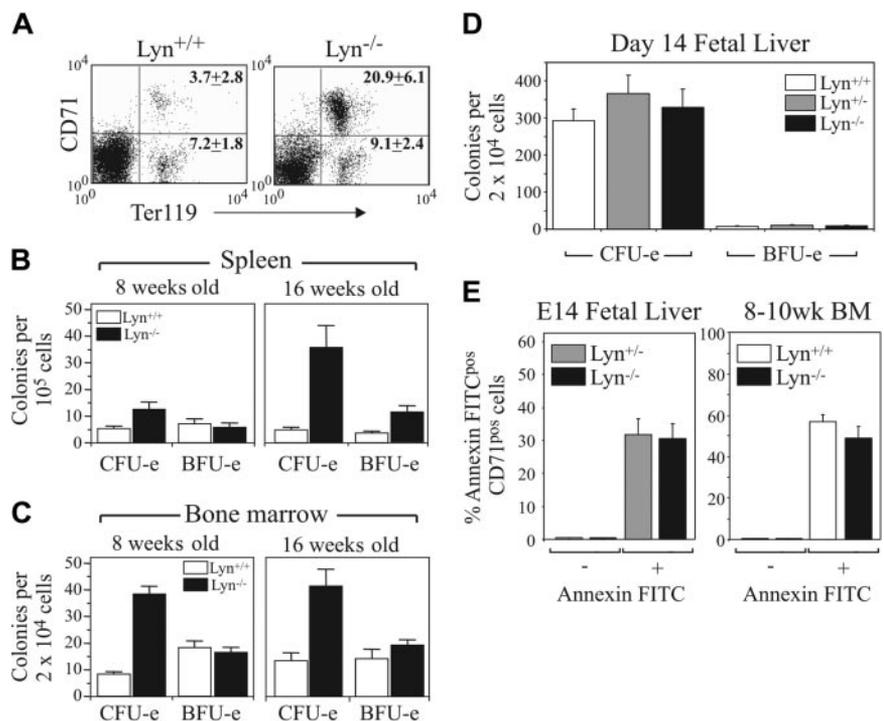


**Figure 1. Normal fetal and bone marrow myelopoiesis but enhanced splenic myelopoiesis in 4- and 16-week-old *Lyn*<sup>-/-</sup> mice.** Progenitors responsive to GM-CSF, IL-3, or M-CSF in the spleen and BM of (A) 16-week-old or (B) 4-week-old sex-matched *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice were assessed by in vitro semisolid agar assays. Data presented in panels A-B correspond to the mean (± SEM) for 2 to 4 experiments using 2 to 3 mice per experiment. (C) Day-14 myeloid fetal liver progenitor populations in *Lyn*<sup>+/+</sup> (n = 9), *Lyn*<sup>+/-</sup> (n = 17), or *Lyn*<sup>-/-</sup> (n = 8) mice were assessed in the presence of the indicated cytokines (mean ± SEM). Fetal livers were derived from day-14 embryos obtained from time-mated *Lyn*<sup>+/+</sup> mice. The splenic myeloid data presented in panel A (left panel) have been reported previously<sup>22</sup> (reprinted from Immunity, Vol 15, Harder KW, Parsons LM, Armes J, et al, "Gain- and loss-of-function *Lyn* mutant mice define a critical inhibitory role for *Lyn* in the myeloid lineage," pages 603-615, copyright (2001), with permission from Elsevier).

(Figure 1A-B). While splenic myelopoiesis was enhanced in 4- and 16-week-old mice, analysis of E14 fetal liver revealed no difference in myeloid progenitor numbers between *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice (Figure 1C).

**Increased numbers of immature erythroid cells and their progenitors in *Lyn*<sup>-/-</sup> mice**

Analysis of histologic sections of spleens from *Lyn*<sup>-/-</sup> mice older than 12 weeks revealed an increase in the number of erythroid cells (not shown). These data, and reports showing increased percentages of Ter119 staining cells in the spleens of *Lyn*<sup>-/-</sup> mice,<sup>38</sup> prompted us to investigate whether erythroid (RBC) development was also perturbed in *Lyn*-deficient mice. We used the transferrin receptor monoclonal antibody (mAb) CD71 and the erythroid lineage-restricted marker Ter119, as described in the characterization of *STAT5A*<sup>-/-</sup> *5B*<sup>-/-</sup> mice,<sup>39-41</sup> to identify erythroblasts in *Lyn*<sup>-/-</sup> spleen. Staining with these Abs revealed a large increase in the proportion of CD71<sup>+</sup>Ter119<sup>+</sup> erythroblasts in the spleens of *Lyn*<sup>-/-</sup> mice (Figure 2A). To investigate whether there was a corresponding increase in primitive erythroid progenitors in the spleens of *Lyn*<sup>-/-</sup> mice, we assessed the numbers of colony- and burst-forming unit erythroid progenitors (CFU-e's and BFU-e's, respectively).<sup>42,43</sup> While there was a slight increase in CFU-e's in the spleens of 8-week-old *Lyn*<sup>-/-</sup> mice, a dramatic increase in CFU-e's became evident by 16 weeks of age (Figure 2B). In the BM, we observed an increase in the number of CFU-e progenitors in both 8- and 16-week-old mice (Figure 2C). By contrast, the numbers of BM primitive BFU-e progenitors were similar at both time points. Similarly, enumeration of BM and spleen mixed erythroid/myeloid colonies generated in a cytokine cocktail of EPO, IL-3, and SCF revealed no difference in the number of these progenitors in 8-week-old *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> BM (*Lyn*<sup>+/+</sup>, 22.3 ± 3.5; *Lyn*<sup>-/-</sup>, 22.5 ± 2.3; n = 12 mice ± SEM/2 × 10<sup>4</sup> cells) or spleen (*Lyn*<sup>+/+</sup>, 2.3 ± 0.6; *Lyn*<sup>-/-</sup>, 3.1 ± 0.5; n = 12 mice ± SEM/10<sup>5</sup> cells). As was the case with fetal liver myeloid



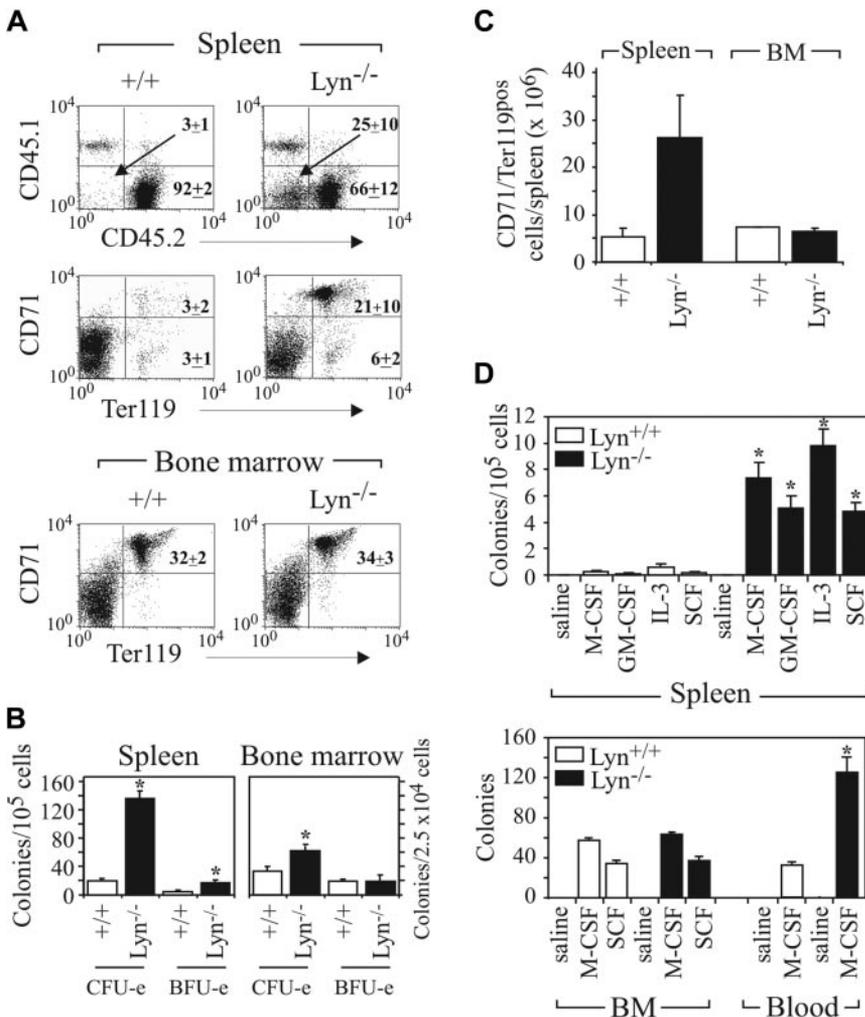
**Figure 2. Expansion of immature erythroid cells in *Lyn*<sup>-/-</sup> spleen and increased erythroid CFU-e numbers in BM and spleen of *Lyn*<sup>-/-</sup> mice.** (A) A 3-color flow cytometric analysis of 8-week-old *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> spleen populations stained with CD71 and Ter119. Propidium iodide (PI)-positive and mature RBCs were excluded from the analysis. Relative percentages (± SD) of immature erythroid (upper right quadrant) and more mature erythroid (lower right) cells of 3 mice of each genotype are indicated. Erythropoiesis in (B) spleen, (C) BM of 8- and 16-week-old *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice, and (D) embryonic day-14 fetal liver was assessed by methylcellulose culture. Mature erythroid (CFU-e) and immature erythroid (BFU-e) progenitors were determined at day 2 and day 10, respectively. (E) Relative percentages (± SD) of annexin-FITC-positive erythroid cells in fetal liver (n = 7-8/genotype) and adult bone marrow (n = 5/genotype) are indicated.

progenitors, no difference in either CFU-e's or BFU-e's was detected in E14 fetal livers (Figure 2D). Additionally, no significant difference in either CD71<sup>+</sup>/Ter119<sup>+</sup> cell numbers or numbers of apoptotic CD71<sup>+</sup> cells were observed in either fetal liver or adult BM (Figure 2E, and not shown). Thus, *Lyn*<sup>-/-</sup> mice develop a significant age-dependent increase in myeloid and erythroid progenitors with the expansion most apparent in relatively mature lineage-restricted progenitors.

### Perturbed hematopoiesis is transplanted with *Lyn*<sup>-/-</sup> bone marrow

To investigate whether the hematopoietic compartment perturbation in *Lyn*<sup>-/-</sup> mice is intrinsic to the stem/progenitor cell, or due to microenvironmental differences, we conducted BM transplantations. Irradiated recipient (Ly5.1) mice received transplants of either *Lyn*<sup>+/+</sup> or *Lyn*<sup>-/-</sup> (Ly5.2) BM. Mice were then analyzed at between 10 to 12 weeks after transplantation for donor cell repopulation and transplantability of perturbed hematopoiesis. Engraftment was assessed by staining peripheral blood, spleen, and BM with the Ly5.2-specific mAb CD45.2 (donor) and Ly5.1 mAb CD45.1 (recipient), which revealed effective reconstitution with donor BM (Figure 3A, and not shown). Analysis of peripheral blood revealed similar percentages of donor-derived CD45.2<sup>+</sup> cells (*Lyn*<sup>+/+</sup>, 94.7 ± 2.4%; *Lyn*<sup>-/-</sup>, 89.3 ± 5.3%; n = 4). However, numbers of CD19<sup>+</sup> B cells in the peripheral blood (*Lyn*<sup>+/+</sup>,

4.2 ± 0.5 × 10<sup>3</sup>/μL; *Lyn*<sup>-/-</sup>, 0.6 ± 0.2 × 10<sup>3</sup>/μL) and spleen (*Lyn*<sup>+/+</sup>, 13.0 ± 1.8 × 10<sup>7</sup>; *Lyn*<sup>-/-</sup>, 2.7 × 10<sup>7</sup> ± 0.4 × 10<sup>7</sup>; n = 6) were dramatically reduced following transplantation with *Lyn*<sup>-/-</sup> BM, as previously described.<sup>20</sup> Interestingly, analysis of recipients of *Lyn*<sup>-/-</sup> BM revealed a significant population of cells in the spleen (25% ± 10%) that failed to stain with either CD45.1 or CD45.2 (Figure 3A). Further analysis of this CD45<sup>-</sup> population revealed that they were CD71<sup>+</sup>Ter119<sup>+</sup> and lacked c-Kit expression, consistent with our previous analysis of the spleens of *Lyn*<sup>-/-</sup> donors (Figures 2A, 3A, and not shown). This population was increased approximately 5-fold in the spleens of *Lyn*<sup>-/-</sup> BM recipients compared with *Lyn*<sup>+/+</sup> transplants. By contrast, the percentages and absolute numbers of CD71<sup>+</sup>Ter119<sup>+</sup> cells in the BM of *Lyn*<sup>-/-</sup> and *Lyn*<sup>+/+</sup> BM recipients were similar (Figure 3A,C). Analysis of erythroid and myeloid progenitors in spleen revealed that, like *Lyn*<sup>-/-</sup> donors, irradiated recipients of *Lyn*<sup>-/-</sup> BM displayed dramatically increased myeloid and erythroid progenitor numbers (Figure 3B,D). Although there was a modest increase in CFU-e's in the BM of *Lyn*<sup>-/-</sup> BM recipients, the number of myeloid progenitors and BFU-e's in the BM was similar (Figure 3B,D). Increased numbers of progenitors responsive to M-CSF in peripheral blood were also detected in the transplants, a phenotype we previously observed in 8-week-old *Lyn*<sup>-/-</sup> mice<sup>22</sup> (Figure 3B,D). Collectively, these results reveal that the age-dependent defect observed in *Lyn*<sup>-/-</sup> donors is recapitulated in *Lyn*<sup>-/-</sup> BM recipients.



**Figure 3. Perturbed myelo/erythropoiesis is transplantable with *Lyn*<sup>-/-</sup> BM.** (A) BM from C57BL/6 Ly5.2 background donor *Lyn*<sup>+/+</sup> or *Lyn*<sup>-/-</sup> mice was transplanted into irradiated C57BL/6 Ly5.1 mice. Engraftment was assessed by identifying donor and recipient cells by FACS analysis with CD45.2 (donor) and CD45.1 (recipient) mAbs. Alternatively, immature erythroid lineage cells in spleen and BM were assessed by staining with CD71 and Ter119 mAbs. Both PI-positive and mature RBCs were excluded from the analysis. The relative percentages of each population (± SD) in 4 recipient mice of each genotype are presented. (B) CFU-e and BFU-e content in the spleen and BM of recipients of either *Lyn*<sup>+/+</sup> or *Lyn*<sup>-/-</sup> BM are shown. (C) The relative number of CD71/Ter119 double-positive erythroid cells in spleen and BM assessed 10 to 12 weeks after transplantation. (D) Myeloid progenitors in spleen, BM, and peripheral blood responsive to the indicated cytokines were determined in mice reconstituted with either *Lyn*<sup>+/+</sup> or *Lyn*<sup>-/-</sup> BM. Progenitors were scored following plating of 10<sup>5</sup> spleen cells, 2.5 × 10<sup>5</sup> BM cells, or 1 to 2 μL peripheral blood. Data presented in panels B and D correspond to the mean (± SEM) for 3 experiments using 2 mice/experiment (\**P* < .01, Student *t* test). Data presented in panel C were obtained from the analysis of 4 mice in 2 experiments (mean ± SEM).

### The hematopoietic defect in *Lyn*<sup>-/-</sup> mice is not B-cell or autoantibody dependent

*Lyn*<sup>-/-</sup> mice have a B-cell defect that results in hyper-IgM and autoantibody production leading to glomerulonephritis and premature death.<sup>34,44</sup> To exclude the possibility that deregulated hematopoiesis is due to underlying autoimmune disease, we eliminated autoantibody production by crossing *Lyn*<sup>-/-</sup> mice with  $\mu$ MT/ $\mu$ MT mice, which have a mutation in the B-cell receptor (BCR)  $\mu$ -chain leading to impaired B-cell maturation.<sup>35</sup> The levels of total immunoglobulin were reduced more than 5000-fold, and antinuclear antibodies were undetectable in *Lyn*<sup>-/-</sup>;  $\mu$ MT/ $\mu$ MT double-mutant mice (Figure 4A-B). However, the number of CD71<sup>+</sup>Ter119<sup>+</sup> cells previously observed in *Lyn*<sup>-/-</sup> mice was not altered in the double mutant, with both *Lyn*<sup>-/-</sup> and *Lyn*<sup>-/-</sup>;  $\mu$ MT/ $\mu$ MT 9-month-old mice containing approximately 8- to 10-fold more splenic erythroid cells (Figure 4C). The total numbers of CFU-e's and BFU-e's in the spleens of *Lyn*<sup>-/-</sup> and *Lyn*<sup>-/-</sup>;  $\mu$ MT/ $\mu$ MT mice were also correspondingly elevated. Likewise, myeloid progenitors were increased in both *Lyn*<sup>-/-</sup> and *Lyn*<sup>-/-</sup>;  $\mu$ MT/ $\mu$ MT mice (Figure 4D-E). Thus, B-cell-dependent autoimmune disease is not a major contributing factor to this phenotype.

### Increased numbers of splenic multipotent progenitors in *Lyn*<sup>-/-</sup> mice

To quantify the number of more primitive progenitor populations, we conducted a day-12 colony-forming unit–spleen assay.<sup>45</sup> While the spleens of *Lyn*<sup>-/-</sup> mice contained approximately 3-fold more CFU-S<sub>12</sub>, the BM contained equivalent numbers of these progenitors (Figure 5A). Thus, in addition to an increase in more mature lineage-restricted progenitors, *Lyn*<sup>-/-</sup> mice possess increased numbers of primitive progenitors in the spleen without a corresponding alteration in BM progenitors.

### Heightened sensitivity of *Lyn*<sup>-/-</sup> mice to 5-FU

The increase in hematopoietic progenitors in *Lyn*<sup>-/-</sup> mice coupled with previous findings illustrating an essential inhibitory signaling role for Lyn within hematopoietic cells led us to speculate that loss of Lyn might enhance the ability of the hematopoietic system to recover from insults that are hematoablative.<sup>46</sup> To test this hypothesis, we challenged mice with the chemotherapeutic drug 5-FU, which is cytotoxic to cells in cycle. The cellularity of the indicated compartments was then assessed at 2-day intervals for 16 days following 5-FU challenge. Surprisingly, the total cellularity of each tissue analyzed was similar irrespective of genotype (Figure 5B-D).

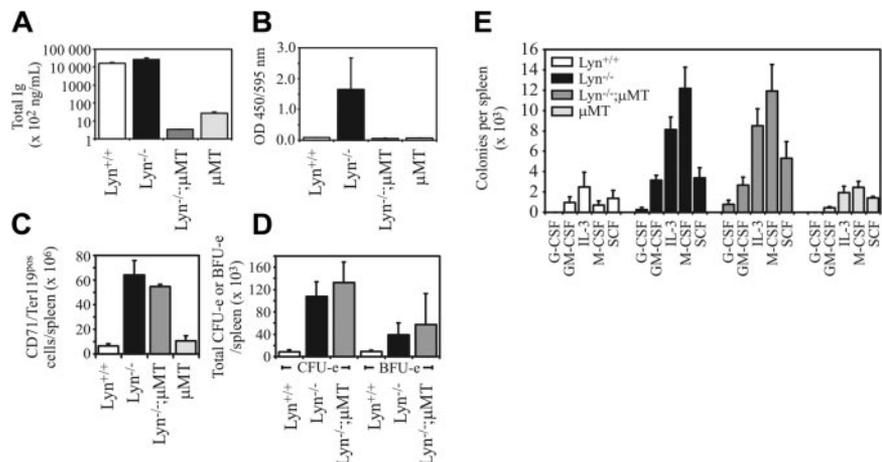
However, 5-FU had a more severe effect on the hematocrit of *Lyn*<sup>-/-</sup> mice than that of control mice (Figure 5E). This, together with the observation that *Lyn*-deficient mice showed a lag of approximately 2 days in the mobilization/expansion of immature CD71<sup>+</sup>Ter119<sup>+</sup> erythroblasts in the spleen following 5-FU treatment (Figure 5F), may suggest a greater percentage of progenitor cells in cycle and thus heightened 5-FU sensitivity. Alternatively, an underlying defect in erythropoiesis in *Lyn*<sup>-/-</sup> mice may lead to an impaired rate of RBC production. To investigate these possibilities we challenged *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice with 5-FU and looked for differential sensitivity of erythroid progenitors to 5-FU. Interestingly, *Lyn*<sup>-/-</sup> mice, which normally contain between 3- to 5-fold more CFU-e's in BM than *Lyn*<sup>+/+</sup> mice (Figure 2C), exhibited approximately 5-fold fewer CFU-e's than *Lyn*<sup>+/+</sup> mice following 5-FU challenge (Figure 5G). However, analysis of BrdU uptake into more mature CD71<sup>+</sup> erythroblasts or Mac-1<sup>+</sup> myeloid cells in untreated mice revealed similar levels of BrdU incorporation (Figure 5H). Thus, *Lyn*<sup>-/-</sup> mice contain an increase in both the absolute number of erythroid progenitors, and an increase in the cycling status of these progenitors, without a corresponding increase in BrdU incorporation into more mature BM erythroblasts.

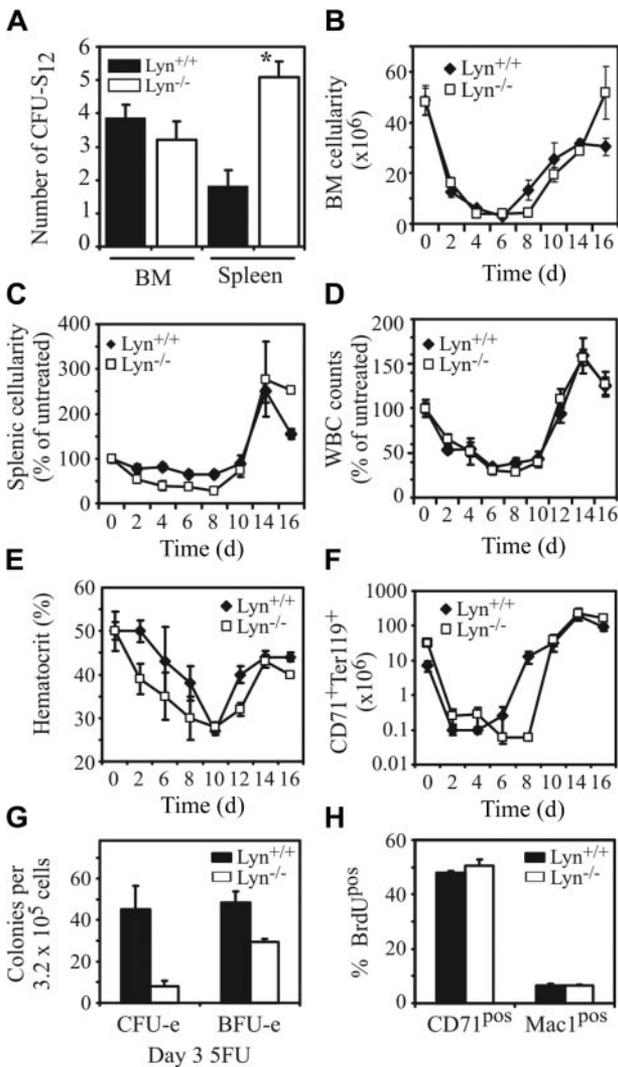
### Similarities between *Lyn*<sup>-/-</sup>, *Me<sup>v</sup>/Me<sup>v</sup>*, and *SHIP-1*<sup>-/-</sup> mice

We and others have defined a relationship between Lyn's inhibitory signaling role and its ability to recruit and activate SHP-1 and SHIP-1 by membrane localization and/or phosphorylation.<sup>20,22,29,30,47</sup> Recent studies have also revealed that Lyn and SHIP-1 act together to negatively regulate M-CSF-dependent AKT activity,<sup>23</sup> a finding supported by our previous results showing that SHIP-1 is a target of Lyn-dependent phosphorylation in primary macrophages and B cells,<sup>22,36</sup> and results illustrating coordinated roles for both Lyn and SHIP-1 in establishing Fc $\gamma$  receptor IIb1 (Fc $\gamma$ RIIb1)-dependent inhibitory signaling in B cells and mast cells.<sup>18,19,24,31</sup> To investigate this relationship further, we analyzed both *Me<sup>v</sup>/Me<sup>v</sup>* and *SHIP-1*<sup>-/-</sup> deficient mice to see if the phenotype of *Lyn*<sup>-/-</sup> mice might be explained by deregulation of either SHP-1 or SHIP-1. We have previously highlighted the similarities between *Lyn*<sup>-/-</sup>, *Me<sup>v</sup>/Me<sup>v</sup>*, and *SHIP-1*<sup>-/-</sup> mice in that they develop splenomegaly, increased splenic and peripheral blood myeloid progenitors, and in the case of *Lyn*<sup>-/-</sup> and *Me<sup>v</sup>/Me<sup>v</sup>* mice, enhanced sensitivity to M-CSF and GM-CSF.<sup>22,32,33</sup>

To further our characterization of *Lyn*<sup>-/-</sup>, *Me<sup>v</sup>/Me<sup>v</sup>*, and *SHIP-1*<sup>-/-</sup> mice, we conducted a limited analysis of the hematopoietic compartments of the 3 mutants and analyzed erythropoiesis in

**Figure 4. B-cell developmental defects and autoimmune disease are not linked to the hematopoietic changes observed in *Lyn*<sup>-/-</sup> mice.** (A) *Lyn*<sup>+/+</sup>, *Lyn*<sup>-/-</sup>, *Lyn*<sup>-/-</sup>;  $\mu$ MT/ $\mu$ MT, and  $\mu$ MT/ $\mu$ MT control mice were assessed for total immunoglobulin levels by ELISA, or (B) antinuclear antibody titers using DNA and nuclear antigen-coated ELISA plates incubated with a 1:500 dilution of sera from mice of the indicated genotypes. Relative optical densities are shown. (C) Total numbers of CD71<sup>+</sup>Ter119<sup>+</sup> cells were enumerated following FACS analysis of spleen cell preparations from mice of the indicated genotypes. (D) Splenic CFU-e's and BFU-e's in mice of the indicated genotypes. Data in panels C-D are means ( $\pm$  SD) for 2 mice per genotype. Mean spleen weights were *Lyn*<sup>+/+</sup>, 0.11  $\pm$  0.01 g; *Lyn*<sup>-/-</sup>, 0.31  $\pm$  0.08 g; and *Lyn*<sup>-/-</sup>  $\mu$ MT, 0.14  $\pm$  0.05 g. (E) Splenic myeloid progenitor numbers responsive to the indicated cytokines in mice of the indicated genotypes. Data represent the mean ( $\pm$  SEM) for 2 mice per genotype per experiment in 2 separate experiments.





**Figure 5.** *Lyn*<sup>-/-</sup> mice have elevated numbers of CFU-S<sub>12</sub> in the spleen but are more severely affected by the cytotoxic drug 5-FU. (A) Numbers of primitive hematopoietic progenitors in the spleen and BM of *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice capable of forming macroscopic colonies in the spleens of irradiated recipient mice 12 days after injection of BM or spleen cell populations (\**P* < .01, Student *t* test). Total cellularity of the (B) BM, (C) spleen, (D) peripheral blood, and (E) hematocrits of *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> was determined at 2-day intervals following intravenous injection of the cytotoxic drug 5-FU. Results for spleen and blood are shown as percent of untreated, since young C57BL/6 background *Lyn*<sup>-/-</sup> mice have diminished splenic and white blood cell (WBC) counts compared with control mice. (F) The total cell number of CD71<sup>+</sup>Ter119<sup>+</sup> double-positive cells in the spleen of 5-FU-treated *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice determined by FACS analysis at 2-day intervals over 16 days. In experiments depicted in panels B-F, 5 age- and sex-matched mice per genotype were analyzed at each time point. Data presented are the mean (± SEM). (G) Numbers of BM CFU-e's and BFU-e's were assessed 3 days after challenge with 5-FU. Data represent the mean (± SD) of 3 to 4 mice/genotype. (H) Percentage BrdU<sup>pos</sup> erythroid (CD71<sup>+</sup>) and myeloid (Mac1<sup>+</sup>) cells in BM 2.5 hours after injection of BrdU (40 mg/kg). Data presented are the mean (± SD) of 3 mice per genotype with similar results observed in 2 independent experiments.

age-matched 6- to 8-week-old mice. Results presented in Table 1 reveal that both *Mev/Mev* and *SHIP-1*<sup>-/-</sup> mice develop similar degrees of splenomegaly, while *Mev/Mev* mice exhibit dramatically reduced BM cellularity and more severe anemia than do age-matched *SHIP-1*<sup>-/-</sup> mice. By contrast, *Lyn*<sup>-/-</sup> mice do not show evidence of splenomegaly until 10 to 13 weeks of age<sup>22</sup> (Table 1). Interestingly, the spleens of *Mev/Mev*, like *Lyn*<sup>-/-</sup> mice, contained a large proportion of CD71<sup>+</sup>Ter119<sup>+</sup> cells (Figure 6A), which, taking into account the splenomegaly in *Mev/Mev* mice, corre-

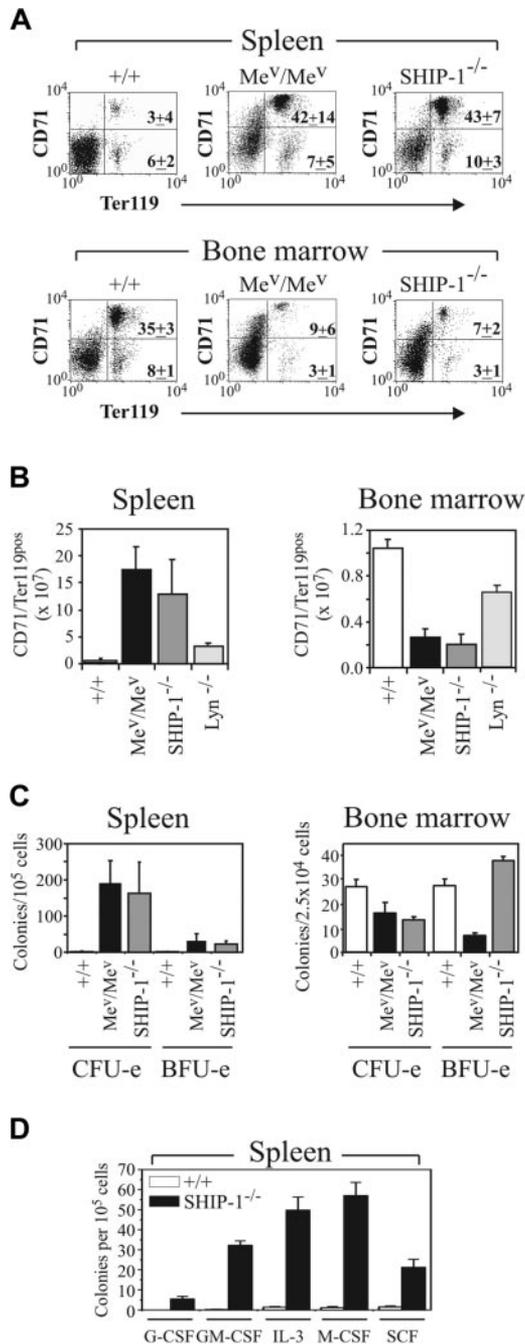
sponded to a 30-fold increase in this population (Figure 6B). Indeed, this population is expanded to a similar degree as that of the Mac-1<sup>+</sup> myelomonocytic/granulocytic population in the spleens of *Mev/Mev* mice (not shown). In keeping with the increase in CD71<sup>+</sup>Ter119<sup>+</sup> cells in the spleen, there was a corresponding increase in splenic CFU-e's, and to a lesser extent, BFU-e's, in *Mev/Mev* mice (Figure 6C), as previously described.<sup>11</sup> Thus, our results showing significant increases in erythroid progenitors in *Lyn*<sup>-/-</sup> mice highlight another similarity between these 2 mouse strains. However, splenic extramedullary hematopoiesis in *Mev/Mev* mice is much more severe than that observed in *Lyn*<sup>-/-</sup> mice of similar age. The BM of *Mev/Mev* mice also exhibited a more dramatic reduction in the proportion and number of CD71<sup>+</sup>Ter119<sup>+</sup> cells than that observed in *Lyn*<sup>-/-</sup> mice, and a corresponding decrease in CFU-e's and BFU-e's, not observed in *Lyn*<sup>-/-</sup> mice (Figure 6A-C).

Surprisingly, analysis of *SHIP-1*<sup>-/-</sup> mice revealed a phenotype very similar to that of *Mev/Mev* mice, and to a lesser degree, *Lyn*<sup>-/-</sup> mice. Staining with CD71 and Ter119 revealed a previously unrecognized increase in erythroblasts in the spleens of *SHIP-1*<sup>-/-</sup> mice. The absolute number of these cells was increased to a similar extent to that observed in 6- to 8-week-old *Mev/Mev* mice (Figure 6A-B), and to a lesser extent, *Lyn*<sup>-/-</sup> mice (Figures 4C, 6B). Analysis of erythroid progenitor numbers revealed a large increase in CFU-e's in spleens of *SHIP-1*<sup>-/-</sup> mice (Figure 6C). Comparison of total CFU-e spleen content revealed *Mev/Mev* 1634 × 10<sup>3</sup> (± 1252 × 10<sup>3</sup>) more than *SHIP-1*<sup>-/-</sup> 490 × 10<sup>3</sup> (± 232 × 10<sup>3</sup>) more than wild-type 6 × 10<sup>3</sup> (± 1 × 10<sup>3</sup>). Like *Mev/Mev* mice, *SHIP-1*<sup>-/-</sup> BM contained a dramatically increased proportion of Mac-1-positive myeloid cells (wild-type, 34.8% ± 1.9%; *SHIP-1*<sup>-/-</sup>, 76.7% ± 5.2%; *Mev/Mev*, 90.3% ± 2.8%; mean ± SD, n = 4/genotype) and a correspondingly reduced proportion and number of CD71<sup>+</sup>Ter119<sup>+</sup> cells (Figure 6A-B). In keeping with this reduction in BM erythroid cells, *SHIP-1*<sup>-/-</sup> mice also have fewer CFU-e progenitors (Figure 6C) as previously reported.<sup>14</sup> In terms of CFU-e content per femur, wild-type mice contained 19 515 (± 2241) progenitors versus 6651 (± 1222) for *SHIP-1*<sup>-/-</sup> mice. By contrast, BM BFU-e content per femur was not significantly different between *SHIP-1*<sup>-/-</sup> and wild-type mice (wild-type, 19 487 ± 1162 vs *SHIP-1*<sup>-/-</sup>, 18 681 ± 2062 per femur) (Figure 6C). The number of progenitors responsive to G-CSF, GM-CSF, IL-3, M-CSF, or SCF was dramatically increased in the spleens of *SHIP-1*<sup>-/-</sup> mice in keeping with previous characterization of GM-CFC content<sup>14</sup> (Figure 6D). Consistent with the increased numbers of myeloid cells in *SHIP-1*<sup>-/-</sup> BM, we observed 2-fold increases in the numbers of BM GM-CSF, M-CSF, and IL-3 CFCs (not shown). Thus, like *Lyn*<sup>-/-</sup> mice, both *SHIP-1*<sup>-/-</sup> and *Mev/Mev* mice develop splenomegaly characterized by increased numbers of immature erythroblasts and myeloid cells in addition to enhanced numbers of their respective progenitors,

**Table 1. Analysis of wild-type, *SHIP-1*<sup>-/-</sup>, *Mev/Mev*, and *Lyn*<sup>-/-</sup> mouse HCT, spleen weight, and BM and spleen cellularity**

Genotype (no. mice)	HCT, %	Spleen weight, g	Spleen cellularity, × 10 <sup>6</sup>	BM cellularity/femur, × 10 <sup>6</sup>
Wild-type (8)	52.4 ± 1.6	0.085 ± 0.012	135 ± 53	17.0 ± 2.1
<i>SHIP-1</i> <sup>-/-</sup> (7)	50.8 ± 4.6	0.213 ± 0.064	300 ± 93	13.4 ± 3.0
<i>Mev/Mev</i> (4-6)	43.7 ± 3.7	0.236 ± 0.091	298 ± 124	4.3 ± 0.9
<i>Lyn</i> <sup>-/-</sup> (5)	50.1 ± 2.4	0.087 ± 0.019	117 ± 9	18.2 ± 1.9

All characterization was performed on mice of 6 to 8 weeks of age. Hematocrit (HCT) was determined by analysis of blood obtained from the retro-orbital venous plexus on an ADVIA 120 hematology system. Values represent the mean ± SD for the number of mice indicated.



**Figure 6. *Motheaten viable* and *SHIP-1*<sup>-/-</sup> mice exhibit a similar but more severe hematopoietic phenotype than *Lyn*<sup>-/-</sup> mice.** (A) FACS profiles of 6- to 8-week-old wild-type (+/+), *Me<sup>v</sup>/Me<sup>v</sup>*, and *SHIP-1*<sup>-/-</sup> spleen and BM populations stained with CD71 and Ter119 mAbs. PI-positive and mature RBCs were excluded from the analysis. Relative percentages of immature erythroid (upper right quadrant) and more mature erythroid (lower right) cells are indicated for 4 mice of each genotype ( $\pm$  SD). (B) Total numbers of CD71<sup>+</sup>Ter119<sup>+</sup> cells in the spleen and BM of +/+, *Me<sup>v</sup>/Me<sup>v</sup>*, *SHIP-1*<sup>-/-</sup>, and *Lyn*<sup>-/-</sup> mice are shown. Values represent the mean ( $\pm$  SD) of 5 C57BL/6 background mice per group, except *Me<sup>v</sup>/Me<sup>v</sup>*, for which 2 mice per group are shown. (C) Mature erythroid (CFU-e) and immature erythroid (BFU-e) progenitors in +/+, *Me<sup>v</sup>/Me<sup>v</sup>*, and *SHIP-1*<sup>-/-</sup> spleen and BM were determined at day 2 and day 10, respectively. Data represent the mean ( $\pm$  SEM) for 2 *Me<sup>v</sup>/Me<sup>v</sup>* mice and 4 +/+ and *SHIP-1*<sup>-/-</sup> mice. (D) Myeloid progenitor content in the spleens of 8-week-old *SHIP-1*<sup>-/-</sup> mice was assessed as in Figure 1 in the presence of the indicated cytokines. Values represent the mean ( $\pm$  SEM) for 4 animals in 2 experiments.

with the latter 2 mutants both more severely affected than *Lyn*<sup>-/-</sup> mice. The BM compartments of the 3 mutants, however, are significantly different both in terms of CD71<sup>+</sup>Ter119<sup>+</sup> erythroid cell numbers and alterations in CFU-e and BFU-e progenitor content.

### *Lyn*<sup>-/-</sup> and *SHIP-1*<sup>-/-</sup> mice exhibit enhanced production of erythroid progenitors but normal recovery of hematocrit following erythropoietic stress

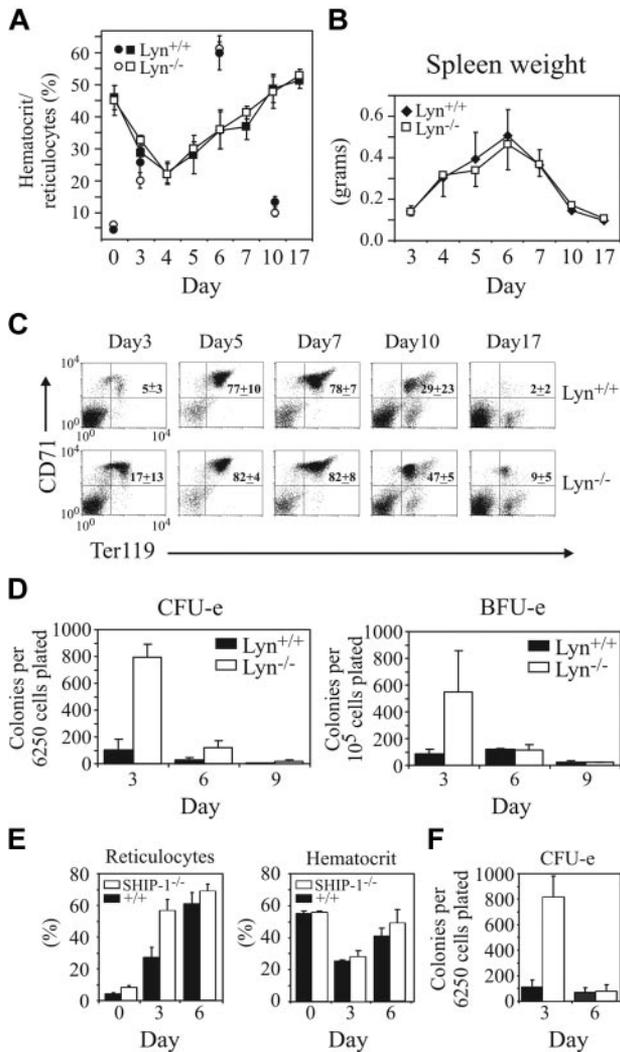
The RBC cytotoxic drug phenylhydrazine induces anemia in mice that is reversed by a wave of erythropoiesis characterized by an accumulation of CD71<sup>+</sup>Ter119<sup>+</sup> cells in the spleen preceding the restoration of normal RBC numbers and hematocrits. A comparison of the response of *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice to phenylhydrazine revealed a similar rate of hematocrit recovery, reticulocyte production, splenomegaly, and increase in splenic CD71<sup>+</sup>Ter119<sup>+</sup> cellularity, despite the fact that *Lyn*<sup>-/-</sup> mice exhibited dramatically increased splenic CFU-e and BFU-e progenitor numbers following phenylhydrazine challenge (Figure 7A-D). Interestingly, comparison of *SHIP-1*<sup>-/-</sup> with *Lyn*<sup>-/-</sup> mice revealed a similar increase in CFU-e's at day 3 after phenylhydrazine challenge in both mutants, although *SHIP-1*<sup>-/-</sup> mice also showed increased numbers of reticulocytes at day 3 (Figure 7E-F). However, this increase in splenic erythroid progenitors and peripheral blood reticulocyte numbers (for *SHIP-1*<sup>-/-</sup> but not *Lyn*<sup>-/-</sup> mice) did not result in a statistically significant enhancement in hematocrit recovery from phenylhydrazine challenge in either strain.

### *Lyn* kinase is dispensable for EPO-dependent activation of Jak/STAT/MAPK pathways and SHIP-1 phosphorylation in primary erythroblasts

Given that the loss of *Lyn* lowers the threshold for B cell,<sup>18-21</sup> mast cell,<sup>25-28</sup> and macrophage stimulation,<sup>22,23</sup> and the reported role for *Lyn* in EPO receptor phosphorylation and STAT5 activation,<sup>48</sup> we investigated whether loss of *Lyn* might modulate EPO-dependent signaling in primary erythroid cells. We purified CD71<sup>+</sup> erythroblasts from spleens of phenylhydrazine-challenged mice and showed that they were predominantly basophilic and early polychromatophilic erythroblasts that expressed both CD71 and Ter119 (Supplemental Figure 1A-B, available by clicking the Supplemental Figure link at the top of the online article on the *Blood* website), but not c-Kit, or CD45 (not shown). Stimulation of these cells with EPO led to dose-dependent tyrosine phosphorylation of both the EPO receptor and Jak2, and corresponding activation of STAT5 and MAP kinases (Supplemental Figure 1C). However, comparison of EPO-dependent signaling in *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> cells revealed no detectable modulation in either the dose response or time course of STAT5 or MAP kinase activation (Supplemental Figure 1D). Moreover, unlike the case in B cells<sup>36</sup> and macrophages,<sup>22</sup> loss of *Lyn* did not alter the tyrosine phosphorylation of SHIP-1 or SHP-1 in erythroblasts (Supplemental Figure 1E, and not shown). Thus, *Lyn* is dispensable for EPO-dependent activation of the JAK/STAT and MAP kinase pathways and the tyrosine phosphorylation of SHP-1 and SHIP-1 in erythroblasts.

## Discussion

*Lyn* plays a critical role in myelomonocytic cells as a regulator of SHP-1, SHP-2, SHIP-1, and the ITIM-containing inhibitory receptors paired immunoglobulin-like receptor B (PIR-B) and signal regulatory protein  $\alpha$  (SIRP $\alpha$ ).<sup>22</sup> Failure to appropriately engage inhibitory signaling in the absence of *Lyn* is thought to underlie the extramedullary hematopoiesis observed in these mice and the hypersensitivity of *Lyn*-deficient macrophages to proliferation and survival signals provided by GM-CSF and M-CSF. Here, we have further defined the temporal progression of perturbations in hematopoiesis that develop as a consequence of *Lyn* deficiency. We have



**Figure 7.** *Lyn*<sup>-/-</sup> mice exhibit normal hematocrit recovery following phenylhydrazine challenge but show enhanced erythroid progenitor expansion. (A) Changes in hematocrit and percentages of reticulocytes over a 17-day period were assessed in *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice after 2 consecutive intraperitoneal injections of phenylhydrazine (60 mg/kg) on days 1 and 2. Mean hematocrit (squares) and reticulocyte (circles) values for 3 to 8 mice per time point ( $\pm$  SD) are shown. (B) Changes in spleen weights in *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice were recorded following phenylhydrazine challenge. Values represent the mean ( $\pm$  SD) for 3 to 5 mice per time point, excepting days 5 to 7, where 6 to 19 mice per point are shown. (C) Representative 2-color FACS profiles of spleen cell populations from phenylhydrazine-treated *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice stained with the mAbs CD71 and Ter119. Mature RBCs and PI-positive cells were excluded from the analysis. (D) Numbers of splenic CFU-e's and BFU-e's in *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice were recorded following phenylhydrazine challenge. Values are the mean ( $\pm$  SD) of 3 to 5 mice per genotype. (E) The response of *SHIP-1*<sup>+/+</sup> and *SHIP-1*<sup>-/-</sup> mice to phenylhydrazine challenge was determined by assessing reticulocyte and hematocrit levels at days 0, 3, and 6 after challenge. Values are the mean ( $\pm$  SD) of 4 mice per genotype. (F) Numbers of splenic CFU-e's in *SHIP-1*<sup>+/+</sup> (■) and *SHIP-1*<sup>-/-</sup> mice (□) were determined 3 and 6 days after phenylhydrazine challenge. Values are the mean ( $\pm$  SD) of 4 mice/genotype.

also identified a novel defect in erythropoiesis that results in a significant increase in erythroid progenitors and erythroblasts in *Lyn*<sup>-/-</sup> spleen. This characteristic becomes more severe with age and coincides temporally with the development of splenomegaly. By contrast, significant increases in splenic myeloid progenitor numbers were apparent in mice as young as 4 weeks of age and preceded splenomegaly.

In light of the superficial similarities between *Lyn*<sup>-/-</sup>, *SHIP-1*<sup>-/-</sup>, and *Mev/Mev* mice, and *Lyn*'s critical role in regulating the tyrosine phosphorylation and plasma membrane mobilization of

*SHIP-1* and *SHP-1*, we compared hematopoiesis in these mice. Interestingly, the composition of the spleens of both *SHIP-1*<sup>-/-</sup> and *SHIP-1*-deficient mice was remarkably similar in terms of the degree of splenomegaly and erythroblast/erythroid progenitor composition. The BM of both mutants also showed a dramatic reduction in immature erythroid cells and an expansion of myeloid populations. Thus, the phenotypes of *SHIP-1*<sup>-/-</sup> and *Mev/Mev* mice were similar, albeit more severe, than similarly aged *Lyn*<sup>-/-</sup> mice. However, it should be noted that *Lyn*<sup>-/-</sup> mice do develop splenomegaly of a similar magnitude to that observed in *Mev/Mev* and *SHIP-1*<sup>-/-</sup> mice as they age.<sup>22</sup> Comparison of *Lyn*<sup>-/-</sup> and *SHIP-1*<sup>-/-</sup> splenic myeloid colony numbers also revealed that myeloid progenitors are dramatically increased in *SHIP-1*<sup>-/-</sup> mice. This increase in myelopoiesis in *SHIP-1*<sup>-/-</sup> mice again is more severe than that observed in *Lyn*<sup>-/-</sup> mice when the degree of splenomegaly is taken into account. There were also notable differences between *Lyn*<sup>-/-</sup>, *Mev/Mev*, and *SHIP-1*<sup>-/-</sup> mice including a diminution in CFU-e and BFU-e (*Mev/Mev*) numbers in the BM not observed in *Lyn*<sup>-/-</sup> mice. However, the dramatic expansion of myeloid populations in the BM of *Mev/Mev* and *SHIP-1*<sup>-/-</sup> mice, and thus potential limitations in stromal-cell support, may be a contributing factor to these BM progenitor population perturbations.

Splenomegaly may arise for a variety of reasons, including loss of signal inhibition, such as in *SHP-1*<sup>-/-</sup><sup>12,13</sup> or *SHIP-1*-deficient mice,<sup>14,15</sup> or be due to the expression of oncogenes such as BCR/Abl.<sup>6,7</sup> Splenomegaly may also reflect an underlying defect resulting in hematologic stress. For example, mice lacking STAT5A/5B<sup>39,40</sup> or Bcl-x<sup>49</sup> exhibit a large increase in splenic erythropoiesis as a compensatory response to impaired RBC production in the BM due to impaired induction of Bcl-X<sub>L</sub> and survival of erythroblasts.<sup>50-52</sup> Impaired terminal differentiation of erythroid cells, such as that observed in mice lacking GATA-1, may also result in splenomegaly due to a dramatic expansion of erythroid progenitors.<sup>53-55</sup> Thus, we reasoned that splenomegaly in *Lyn*<sup>-/-</sup> mice might be due to *Lyn*'s role in signal inhibition, or an as-yet-unrecognized role for *Lyn* in the production, survival, or differentiation of erythroblasts. Severe defects in erythropoiesis in mice are most readily apparent during fetal development, a period of high erythropoietic rate.<sup>39</sup> However, analysis of fetal liver hematopoiesis in *Lyn*<sup>-/-</sup> mice failed to show alterations in either myeloid or erythroid progenitor numbers, and quantitation of neonatal erythroblasts revealed no significant difference between *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice (not shown). This is in keeping with our previous analysis of aged *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice, which failed to show evidence of anemia.<sup>22</sup> A thorough analysis of apoptosis in immature erythroblasts in adult BM and embryonic day-14 fetal liver also revealed no differences between *Lyn*<sup>+/+</sup> and *Lyn*<sup>-/-</sup> mice, in keeping with our finding that *Lyn* deficiency does not alter EPO-dependent STAT5 phosphorylation. However, the BM of *Lyn*<sup>-/-</sup> mice did display a modest decrease in immature erythroid cells and an increase in CFU-e's, suggesting an impairment in the expansion/differentiation of CFU-e progenitors into erythroblasts. Interestingly, Helgason et al<sup>14</sup> have speculated that the diminution of BM CFU-e's and Ter119<sup>+</sup> cells in *SHIP-1*<sup>-/-</sup> BM may indicate a positive role for *SHIP-1* in erythropoiesis. Our data suggesting an underlying defect in erythropoiesis in *Lyn*<sup>-/-</sup> mice were supported by our finding that 5-FU-dependent anemia<sup>56</sup> was more severe, and the anemia-induced expansion of the splenic CD71<sup>+</sup>/Ter119<sup>+</sup> erythroblasts was delayed in *Lyn*<sup>-/-</sup> mice. However, the heightened sensitivity of *Lyn*-deficient mice to 5-FU was likely due to a greater proportion of cycling *Lyn*<sup>-/-</sup> progenitor cells, as assessment of CFU-e and BFU-e erythroid progenitor numbers 3 days after 5-FU

challenge revealed a more severe diminution of erythroid progenitors in *Lyn*<sup>-/-</sup> versus *Lyn*<sup>+/+</sup> mice. Interestingly, the hematopoietic progenitor cell compartment in *SHIP-1*<sup>-/-</sup> mice also exhibits heightened sensitivity to 5-FU and diminished stem cell competitive repopulation ability.<sup>57</sup>

The similarity between *Lyn*<sup>+/+</sup>, *Lyn*<sup>-/-</sup>, and *SHIP-1*<sup>-/-</sup> mice in hematocrit recovery following treatment with the RBC-depleting drug phenylhydrazine argues against a major defect in erythropoiesis in either *Lyn*<sup>-/-</sup> or *SHIP-1*<sup>-/-</sup> mice. Indeed, assessment of erythroid progenitor numbers in the spleens of *Lyn*<sup>+/+</sup>, *Lyn*<sup>-/-</sup>, and *SHIP-1*<sup>-/-</sup> mice after phenylhydrazine treatment revealed a more robust expansion of this lineage in both *Lyn*<sup>-/-</sup> and *SHIP-1*<sup>-/-</sup> mice. It is surprising, however, that the large increase in erythroid progenitors in the spleens of both *Lyn*<sup>-/-</sup> and *SHIP-1*<sup>-/-</sup> mice did not ultimately lead to an enhanced recovery from phenylhydrazine. Indeed, this observation raises the possibility that *Lyn* and *SHIP* may act as negative regulators during early myelo/erythropoiesis, but may be required for appropriate differentiation of hematopoietic cells at later stages of development.

Given the similarities between *Lyn*<sup>-/-</sup>, *Me<sup>v</sup>/Me<sup>v</sup>*, and *SHIP-1*<sup>-/-</sup> mice, together with studies showing a role for *Lyn* in EPO-dependent signaling,<sup>48</sup> and the reported role of SHP-1 as a direct antagonist of EPO receptor and Jak2 phosphorylation/activation,<sup>58,59</sup> we investigated the role of *Lyn* in EPO receptor signaling in primary erythroblasts. We found no significant difference in either the dose response or time course of activation of STAT5 or MAP kinase following EPO stimulation. Surprisingly, *Lyn* was also dispensable for both *SHIP-1* and *SHP-1* (not shown) tyrosine phosphorylation in these cells. Thus, *Lyn* does not play a significant role in the EPO-dependent activation of the JAK/STAT or MAP kinase pathways, or in *SHP-1* and *SHIP-1* tyrosine phosphorylation

in erythroblasts at this stage of development. These results suggest that the increased erythropoiesis observed in *Lyn*<sup>-/-</sup> mice is unlikely to be a simple consequence of heightened EPO sensitivity of *Lyn*<sup>-/-</sup> erythroblasts. However, *Lyn*-dependent regulation of other EPO-dependent signaling pathways, or regulation of EPO signaling at other stages of erythroid development, cannot be ruled out. Inhibitory roles for *Lyn* in signaling from other receptors expressed within this lineage, functions for *Lyn* in differentiation or mobilization of erythroid cells,<sup>60-62</sup> and roles for *Lyn* in more primitive progenitor populations also remain to be explored. Regardless, it is clear that *Lyn* plays a critical role in signaling required for appropriate development of the myeloid, erythroid, and B-lymphoid arms of the hematopoietic system. Analysis of combined loss-of-function mutants of *Lyn*, *SHP-1*, and/or *SHIP-1* will be an important step in assessing the genetic interactions between these signal transduction regulators in hematopoietic stem/progenitor cell biology.

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## Erratum

In the article by Vogt et al entitled "Heparan sulfate on endothelial cells mediates the binding of *Plasmodium falciparum*-infected erythrocytes via the DBL1 $\alpha$  domain of PfEMP1," which appeared in the March 15, 2003, issue of *Blood* (Volume 101:2405-2411), the Figure 4B legend should begin, "DBL1 $\alpha$ -GST (□) and GST alone (●) . . . ."