By Nobuko Uchida, Hector L. Aguila, William H. Fleming, Libuse Jerabek, and Irving L. Weissman

Hematopoietic stem cells (HSCs) are believed to play a critical role in the sustained repopulation of all blood cells after bone marrow transplantation (BMT). However, understanding the role of HSCs versus other hematopoietic cells in the quantitative reconstitution of various blood cell types has awaited methods to isolate HSCs. A candidate population of mouse HSCs, Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells, was isolated several years ago and, recently, this population has been shown to be the only population of BM cells that contains HSCs in C57BL/Ka-Thy-1.1 mice. As few as 100 of these cells can radioprotect 95% to 100% of irradiated mice, resulting in long-term multilineage reconstitution. In this study, we examined the reconstitution potential of irradiated mice transplanted with purified Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> BM cells. Donor-derived peripheral blood (PB) white blood cells were detected as early as day 9 or 10 when 100 to 1,000 Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells were used, with minor dose-dependent differences. The reappearance of platelets by day 14 and thereafter was also seen at all HSC doses (100 to 1,000 cells), with a slight dose-dependence. All studied HSC doses also allowed RBC levels to recover, although at the 100 cell dose a delay in hematopoietic recovery was observed at day 14. When irradiated mice were transplanted with 500 Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells compared with 1 × 10<sup>5</sup> BM cells (the equivalent amount of cells that contain 500 Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells as well as progenitor and mature cells), very little difference in the kinetics of recovery of PB, white blood cells, platelets, and hematocrit was observed. Surprisingly, even when 200 Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells were mixed with 4 × 10<sup>5</sup> Sca-1<sup>−</sup> BM cells in a competitive repopulation assay, most of the early (days 11 and 14) PB myeloid cells were derived from the HSC genotype, indicating the superiority of the Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells over Sca-1<sup>−</sup> cells even in the early phases of myeloid reconstitution. Within the Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> population, the Rhodamine 123 (Rh123)<sup>−</sup> subset dominates in PB myeloid reconstitution at 10 to 14 days, only to be overtaken by the Rh123<sup>+</sup> subset at 3 weeks and thereafter. These findings indicate that HSCs can account for the early phase of hematopoietic recovery, as well as sustained hematopoiesis, and raise questions about the role of non-HSC BM populations in the setting of BMT.

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Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells, with only a brief lag in comparison to BM cells. Since submission of this report, a similar study by Okada et al<sup>25</sup> have reported results showing that Lin<sup>−</sup>c-kit<sup>−</sup>Rh123<sup>−</sup> cells, about 10-fold less enriched for radioprotective cells than Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells, also give rise to early appearing myelomonocytic and B-lineage blood cells.

**MATERIALS AND METHODS**

**Mouse strains.** The C57BL/6 (Thy-1.2, Ly-5.2), C57BL/Ka (Thy-1.2, Ly-5.2), C57BL/6J-Ly-5.1-Pep<sup>z</sup> (Thy-1.2, Ly-5.1), C57BL/Ka-Thy-1.1 (Thy-1.1, Ly-5.2), and C57BL/Ka-Thy-1.1-Ly-5.1 (Thy-1.1, Ly-5.1) congenic mouse strains (Ly-5 nomenclature defined in Morse et al<sup>25</sup>; see Table 2) used in the study were bred and maintained in the mouse facility at Stanford University. All mice were regularly maintained on acidified water (pH 2.5).

**Purification of Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells.** BM cells were obtained by flushing tibias and femurs. Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells were stained and isolated from BM as described previously.<sup>18</sup> The following lineage marker antibodies were used: RA3-6B2 for the B-lineage marker B220; for T-cell markers, CD4 (GK1.5) and CD8 (53.6.72); for myelomonocytic markers, GR-1 (RB6-8C5) and Mac-1 (M1/70.15.11.5); and for erythrocytes, TER-119 (Kina et al, manuscript, in preparation). In some experiments, antibodies for NK1.1 (PK136; PharMingen, San Diego, CA) and CD5 (53.7.3) were included for lineage markers. Antibodies used for positive selection were 19X6E5 (Thy-1.1) and Sca-1 (Ly-6E). The cells were incubated for 20 minutes on ice for each step, followed by a wash through a fetal calf serum cushion. After the final wash, cells were resuspended in Hanks’ Balanced Salt Solution (HBSS) containing 1 μg/mL propidium iodide. The labeled cells were analyzed and sorted with a dual-laser FACS (Beckton Dickinson Immunocytometry Systems, Mountain View, CA), modified as described and made available through the FACS shared user group at Stanford University. Dead cells were excluded from analysis by their propidium iodide staining characteristics. After sorting, the purity of Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells was checked by reanalysis for NK1.1. For FACS, the Lin<sup>−</sup>Sca-1<sup>−</sup> subset will be called Lin<sup>+</sup> hereafter, although it includes CD4<sup>+</sup> and Mac-1<sup>+</sup> cells (Morrisson and Weissman, manuscript in preparation).

**HSCs and BMT.** Recipient mice were lethally irradiated by a 250-kV X-ray machine in two split doses with a 3-hour interval. Later the same day, sorted Thy-1.1<sup>+</sup>Lin<sup>−</sup>Sca-1<sup>−</sup> cells or BM cells were injected (200 μL/mouse) intravenously into the retroorbital plexus. After irradiation, mice were maintained on antibiotic water containing 10<sup>4</sup> U/L of polymyxin B sulfate and 1.1 g/L of neomycin sulfate.

**Peripheral blood (PB) cells and BM analysis.** Experimental animals were analyzed 7 to 28 days after BMT. PB was obtained (250 μL/mouse) from the retroorbital sinus. Two to four mice from each group were used, and same mouse was bled at only one time point in most cases. White blood cell (WBC) counts, platelet counts, and hematocrits were determined by CELL-DYN 1600 (Sequoia-Turner, Mountain View, CA), and were confirmed manually with the use of Unopette (Becton Dickinson, Rutherford, NJ).

The significance of differences of hematopoietic recovery between different doses of HSCs transplanted was determined by the two-tailed z-test using the StatView II (Abacus Concepts, Berkeley, CA) on a Macintosh Icx (Apple Computer, Cupertino, CA), and in some cases by χ<sup>2</sup> analysis.

**PB leukocytes (PBLs) were analyzed by immunofluorescence staining and FACS analysis were performed as described previously.<sup>30</sup> Two-color staining was performed with the above mentioned lineage markers. Allelic markers for donor/host T cam Tai–Thy-1.1, antibody 19X6E5, anti-Thy-1.2; purchased from Caltag, South San Francisco, CA) cells were used along with allelic markers specific for donor hematopoietic cells (anti-Ly-5.2, antibody ALI4-A2).

Hemato poetic loss in lethally irradiated mice. We determined the WBC count, platelet count, and hematocrit to monitor hematologic changes in lethally irradiated mice. Two different congenic mouse strains were tested for hematologic loss after lethal irradiation. C57BL/6-Ly-5.1 mice were irradiated at 8.5 Gy (Fig 2B, shaded lines) and C57BL/Ka mice were irradiated at 8.8 GY (Fig 2C, shaded lines). Irradiated mice that did not receive any cells died 7 to 14 days after the irradiation because of hematopoietic failure. The WBC count showed a sharp decrease by day 1 after irradiation, from 6 to 7 × 10<sup>6</sup> cells/μL to levels of less than 1 × 10<sup>3</sup> cells/μL. On the other hand, platelet counts stayed relatively high for 3 days (900 to 1,200 × 10<sup>3</sup>/μL) after irradiation, followed by a rapid decline 5 to 7 days after irradiation to less than 100 × 10<sup>3</sup>/μL. Strain differences were observed for the decline of hematocrit. The hematocrit decreased constantly from 43% to 29% at day 12 (1.1% decline per day) for C57BL/Ka mice, and from 44% to 6% (2.7% decline per day) at day 14 for C57BL/6-Ly-5.1 mice, although C57BL/Ka mice were irradiated at a slightly higher dose. It shall be of interest to test whether red blood cell (RBC) lifespan in these two strains of mice differ in a genetically related fashion.
Hematopoietic recovery in lethally irradiated mice transplanted with Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cells. We tested whether the Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> population of cells was sufficient for rapid reconstitution of PB cells. Hematopoietic recovery in lethally irradiated mice was monitored over a period of 28 days after transplantation with 100, 500, or 1,000 Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cells (Figs 2 and 3). Recovery of PB WBC levels greater than irradiation controls was not seen at day 7 with these cells, but was clearly above background at the next interval tested, day 14, and thereafter. In two of three experiments, hosts receiving 100 cells had lower WBC counts than hosts receiving 500 or 1,000 cells on day 14, but not thereafter (Fig 2). At no time point did hosts receiving 1,000 cells have higher WBC counts than hosts receiving 500 cells. Therefore, 500 cells appears to be the "saturating dose" of HSCs as measured by WBC counts. At day 28, the WBC levels in the recipient mice had increased to 1 to 3 × 10<sup>5</sup> cells/μL for all Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cell doses.

The nadir of platelet counts occurred at day 7 in irradiated mice reconstituted with 1,000 Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cells (Fig 2). By day 10 to 14 (and thereafter), the platelet counts
reached levels above background ($>50 \times 10^3/\mu L$) in all mice receiving doses of Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cell transplants (Fig 2). In all three experiments, the platelet counts at day 14 were directly correlated with the input Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cell number, but in two of three experiments at day 21, mice receiving 100 cells had much higher platelet counts than mice receiving 500 cells. Thus, in mice receiving Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cell transplants of 100 to 1,000 cells, a thrombocytopenia of less than $100 \times 10^3/\mu L$ is overcome between 14 and 21 days after irradiation/reconstitution.

Recovery of the hematocrit was also correlated with the number of Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cells transplanted. A greater decrease in hematocrit was observed when only 100 Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cells were transplanted. In these cases, the nadir occurred at day 14. In contrast, very few mice that received either 500 or 1,000 Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cells showed a decrease in the hematocrit to less than 30%.

The data from three independent experiments were combined in Fig 3. The kinetics of WBC recovery in these mice transplanted with different doses of HSCs did not differ significantly (Fig 3). Although the levels of WBCs in hosts transplanted with 500 Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cells were greater than hosts transplanted with 100 Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cells on day 14, they were not statistically different (see Fig 3 legend).

On the other hand, the platelet counts at day 14 were directly correlated with the dose of Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cells transplanted (Fig 3). At day 14, the platelet numbers were significantly different statistically between 100 cells and 500 cells transplanted, as well as between 500 cells and 1,000 cells transplanted. However, the platelet counts by day 21 were similar with 100 cells or 500 cells transplanted, and even between 100 cells and 1,000 cells transplanted.

The hematocrit nadir occurred at day 14. Although we observed statistically significant differences at that time in hematocrits for mice that received 100 Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cells versus 500 Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cells, no difference was observed in hematocrits in the mice that received 500 cells and 1,000 cells at day 14. The hematocrits of all groups were similar by day 21 and thereafter (Fig 3).

The early phase of hematopoietic recovery after transplantation of Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cells is donor-derived. To test whether Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ HSC transplantation led to donor-derived hematopoietic recovery in lethally irradiated mice, Ly-5 congenic marked B, myeloid, and T cells were examined in mice from experiment 1 on day 14 (Fig 4). The numbers of PBLs were still low at the time point (Figs 2 and 3). This was particularly true in those mice transplanted with 100 Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cells. We could analyze only $\sim$1,000 cells per sample. As expected for this early time point, very low levels of B cells were detected ($<6\%$ of PBLs) from mice that received either 100 or 500 Thy-1.1$^{b}$ Lin$^{-}$ Sca-1$^{-}$ cells, whereas 10% to 20% PBLs analyzed were B220$^{+}$ cells from mice that received 1,000 cells.
It was shown previously that 100 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells could radioprotect 95% to 100% of lethally irradiated mice. Although 3 of 3 mice tested receiving 100 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells contained donor-derived myeloid cells (65% of Mac-1 and Gr-1<sup>−</sup> blood cells) on day 14 after transplantation, I of 3 mice did not contain donor-derived B cells at this time point (Fig 4A). About 10% to 20% of PBLs were myeloid, and ≈93% of these were donor-derived in the mice transplanted with 500 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells (Fig 4B). When 1,000 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells were transplanted, 20% to 30% of PBLs were myeloid and ≈94% of these were donor-derived (Fig 4C). At 14 days, we detected not only myeloid but also B cells derived from the donor in animals receiving 1,000 cells (Fig 4C).

The mice studied in Figs 2 and 3 were kept alive to provide serial sampling intervals from each cohort. Unfortunately, at days 7 to 14 after irradiation and reconstitution it was difficult to obtain sufficient amounts of blood to evaluate WBCs, hematocrit, and platelets, as well as to stain for Ly-5 (donor versus host) markers of blood cell type without compromising their well-being or viability. Therefore, a cohort of mice reconstituted with 100 to 500 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> BM cells was prepared to examine donor repopulation of the BM. A representative sample of BM from a mouse 9 days after lethal irradiation and injection with 200 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells was examined at 29 weeks after transplantation. Normal levels of B, myeloid, and T cells of donor origin were detected in the PB of lethally irradiated animals whether they had received 100 or 1,000 cells (Fig 6). Virtually all B cells (≥98%) and myeloid cells (≥98%) were donor-derived in mice transplanted with 1,000 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells, whereas at this time point 59% of T cells were donor-derived.

We detected endogenous T cells in the blood of mice transplanted with 1 × 10<sup>5</sup> BM cells as well, presumably because a subset of T cells is radioreistant. Three of four mice transplanted with 100 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells were long-term reconstituted with donor-derived cells in multilineages. The low dose of irradiation received by these mice (7.8 Gy for experiment 1), due to x-ray machine calibration error.

The long-term hematopoietic recovery after transplantation of Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells is donor-derived. To test whether Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> HSC transplantation led to long-term donor-derived multilineage reconstitution, PB cells were examined at 29 weeks after transplantation. Normal levels of B, myeloid, and T cells of donor origin were detected in the PB of lethally irradiated animals whether they had received 100 or 1,000 cells (Fig 6). Virtually all B cells (≥98%) and myeloid cells (≥98%) were donor-derived in mice transplanted with 1,000 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells, whereas at this time point 59% of T cells were donor-derived. We detected endogenous T cells in the blood of mice transplanted with 1 × 10<sup>5</sup> BM cells as well, presumably because a subset of T cells is radioreistant. Three of four mice transplanted with 100 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells were long-term reconstituted with donor-derived cells in multilineages. The low dose of irradiation received by these mice (7.8 Gy for experiment 1), due to x-ray machine calibration error,
Table 1. Reconstitution of Irradiated Ly-5 Congenic Mice With Whole BM or Purified Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> Cells

<table>
<thead>
<tr>
<th>Days After Injection</th>
<th>No. of Cells Injected</th>
<th>n</th>
<th>Percentage of Donor Cells</th>
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<tr>
<td>Experiment 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>100 Thy-1.1&lt;sup&gt;+&lt;/sup&gt; Lin&lt;sup&gt;-&lt;/sup&gt; Sca-1&lt;sup&gt;-&lt;/sup&gt;</td>
<td>4</td>
<td>36.2 ± 2.4</td>
</tr>
<tr>
<td>Day 14</td>
<td>500 Thy-1.1&lt;sup&gt;+&lt;/sup&gt; Lin&lt;sup&gt;-&lt;/sup&gt; Sca-1&lt;sup&gt;-&lt;/sup&gt;</td>
<td>4</td>
<td>96.5 ± 1.3</td>
</tr>
<tr>
<td>Day 21</td>
<td>500 Thy-1.1&lt;sup&gt;+&lt;/sup&gt; Lin&lt;sup&gt;-&lt;/sup&gt; Sca-1&lt;sup&gt;-&lt;/sup&gt;</td>
<td>4</td>
<td>99.1 ± 0.2</td>
</tr>
<tr>
<td>Day 28</td>
<td>500 Thy-1.1&lt;sup&gt;+&lt;/sup&gt; Lin&lt;sup&gt;-&lt;/sup&gt; Sca-1&lt;sup&gt;-&lt;/sup&gt;</td>
<td>4</td>
<td>97.3 ± 0.8</td>
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<tr>
<td>Experiment 5</td>
<td></td>
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<tr>
<td>Day 14</td>
<td>500 Thy-1.1&lt;sup&gt;+&lt;/sup&gt; Lin&lt;sup&gt;-&lt;/sup&gt; Sca-1&lt;sup&gt;-&lt;/sup&gt;</td>
<td>4</td>
<td>78.1 ± 2.8</td>
</tr>
<tr>
<td>Day 21</td>
<td>500 Thy-1.1&lt;sup&gt;+&lt;/sup&gt; Lin&lt;sup&gt;-&lt;/sup&gt; Sca-1&lt;sup&gt;-&lt;/sup&gt;</td>
<td>4</td>
<td>91.4 ± 2.2</td>
</tr>
<tr>
<td>Day 28</td>
<td>500 Thy-1.1&lt;sup&gt;+&lt;/sup&gt; Lin&lt;sup&gt;-&lt;/sup&gt; Sca-1&lt;sup&gt;-&lt;/sup&gt;</td>
<td>4</td>
<td>94.1 ± 2.3</td>
</tr>
<tr>
<td>Day 35</td>
<td>500 Thy-1.1&lt;sup&gt;+&lt;/sup&gt; Lin&lt;sup&gt;-&lt;/sup&gt; Sca-1&lt;sup&gt;-&lt;/sup&gt;</td>
<td>4</td>
<td>94.9 ± 1.4</td>
</tr>
<tr>
<td>Day 14</td>
<td>1 x 10&lt;sup&gt;6&lt;/sup&gt; whole BM</td>
<td>3</td>
<td>84.3 ± 3.5</td>
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<tr>
<td>Day 21</td>
<td>1 x 10&lt;sup&gt;6&lt;/sup&gt; whole BM</td>
<td>3</td>
<td>93.7 ± 1.7</td>
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<tr>
<td>Day 28</td>
<td>1 x 10&lt;sup&gt;6&lt;/sup&gt; whole BM</td>
<td>3</td>
<td>94.8 ± 1.6</td>
</tr>
<tr>
<td>Day 35</td>
<td>1 x 10&lt;sup&gt;6&lt;/sup&gt; whole BM</td>
<td>3</td>
<td>96.9 ± 1.1</td>
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Mice were lethally irradiated (10 Gy) and injected with 1 x 10<sup>6</sup> whole BM or 100 or 500 Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> cells. BM of the reconstituted mice was analyzed at several time points, as indicated. Two-color staining was performed with antibodies specific for donor (anti-Ly-5.2, antibody ALI-4A2) and host (anti-Ly-5.1, antibody A20.1) hematopoietic cells. At day 7, there were significant autofluorescent cells that were positive for both Ly-5.1 and Ly-5.2 markers. We excluded these cells from calculation for the percentage of donor cells in the BM. However, such double-positive cells were not detected at day 14 or thereafter.

would have not been expected to eliminate all host BM cells. Among three mice tested, 91%, 55%, and 35% of B, myeloid, and T cells, respectively, were donor-derived. Thus, long-term multilineage reconstitution as well as early phase hematopoietic recovery was achieved with Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> cells.

Comparison of the kinetics of hematopoietic recovery with purified Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> HSCs with that achieved with unfractionated BM containing equivalent numbers of Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> HSCs. We compared hematopoietic recovery with purified Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> HSCs with BM that contains similar numbers of Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> HSCs.

Fig 6. Long-term multilineage reconstitution by transplanting purified Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> cells. The recipient mice were transplanted with 100 (A) or 1,000 (B) Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> cells. PB cells were analyzed 29 to 32 weeks after irradiation/transplantation. The percentages of Ly-5<sup>-</sup>marked B220<sup>+</sup>, Mac-1 and Gr-1<sup>-</sup>, or Thy-1<sup>-</sup> cells originating from transplanted Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> cells in a representative mouse are indicated in each panel. Three of four mice transplanted with 100 Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> cells were reconstituted with long-term multilineage donor-derived cells. Averages of 91% ± 6%, 56% ± 24%, and 35% ± 11% of donor-derived B, myeloid, and T cells, respectively, were detected in the blood of mice that received 100 Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>-</sup> cells (n = 3), 98% ± 0.5%, 98% ± 0.4%, and 59% ± 11% donor cells in mice that received 1,000 cells (n = 2).
compared hematopoietic recovery with 500 Thy-1.1 'Lin-' Sca-1 ' cells to that achieved with BM containing equivalent numbers of HSCs. Mice were lethally irradiated and injected with 500 Thy-1.1 'Lin-' Sca-1 ' cells or 1 x 10^6 BM cells. The data were combined from experiments 2 and 3 from Fig 2 (A) and experiment 5 (B). The normal values of WBC counts, platelet counts, and percentages of hematocrit of unirradiated mice were represented (shaded box). Data represent the mean ± SE obtained from 5 to 9 (A) and 3 to 4 (B) samples from mice transplanted with 500 Thy-1.1 'Lin-' Sca-1 ' cells or 1 x 10^6 BM cells in each data point. Platelet counts were not determined in experiment 5. **WBC at day 28, P < .01. **Platelets at day 14, P < .002. **Platelets at day 28, P < .005. ****Hematocrit at day 14, P < .005. *Hematocrit at day 36, P < .01.

as well as mature cells and lineage-committed progenitors. Non-HSC BM cells, representing 99.95% of BM, contain various stages of mature cells and committed progenitors, including cells that give rise to short-term cobblestone-forming clusters in Dexter culture, most in vitro colony-forming cells (CFCs), as well as day-8 colony-forming units-spleen (CFU-S) and some day-12 CFU-S. 

Non-HSC BM cells, representing 99.95% of BM, contain various stages of mature cells and committed progenitors, including cells that give rise to short-term cobblestone-forming clusters in Dexter culture, most in vitro colony-forming cells (CFCs), as well as day-8 colony-forming units-spleen (CFU-S) and some day-12 CFU-S. It would be reasonable to assume that these progenitors may play a major role in the early phases of hematopoietic engraftment in BMT, because they are capable of differentiating to mature blood cells within a few days. Thy-1.1 'Lin-' Sca-1 ' cells represent ~0.05% of BM; 500 Thy-1.1 'Lin-' Sca-1 ' cells would be calculated to be contained in 1 x 10^6 BM cells. Thus, we compared hematopoietic recovery with 500 Thy-1.1 'Lin-' Sca-1 ' and 1 x 10^6 transplanted BM cells (Fig 7). Surprisingly, 1 x 10^6 BM cells, the mixture of mature and immature hematopoietic cells including ~500 Thy-1.1 'Lin-' Sca-1 ' cells, had little additional effect in comparison to 500 Thy-1.1 'Lin-' Sca-1 ' cells on the outcome of early reconstitution of WBCs, platelets, and hematocrit. WBC recovery in mice receiving 500 Thy-1.1 'Lin-' Sca-1 ' cells or 1 x 10^6 BM cells was similar until day 14. However, in two of three experiments, this increase of WBC count of mice receiving 500 Thy-1.1 'Lin-' Sca-1 ' cells did not keep pace over the next 2 weeks with that achieved by reconstitution with 1 x 10^6 BM, although in the third experiment it did (Fig 7). The PB platelet counts in mice injected with 500 Thy-1.1 'Lin-' Sca-1 ' cells was delayed only 1 to 4 days longer than those achieved by 1 x 10^6 BM cells. Mice receiving 1 x 10^6 BM cells sustained a hematocrit greater than 32%, similar to those receiving 500 Thy-1.1 'Lin-' Sca-1 ' cells. Only a slight delay in hematocrit recovery was observed at day 14. Therefore, in these experiments, the early postirradiation recovery of WBCs (mainly myelomonocytic cells), platelets, and RBCs is comparable to that achieved with 2,000-fold more (1 x 10^6) whole BM cells.

Competitive reconstitution of 200 Thy-1.1 'Lin-' Sca-1 ' cells versus 4 x 10^6 co.injected Sca-1 ' cells. We wished to compare, in the same host, the reconstitution capacity of 200 Thy-1.1 'Lin-' Sca-1 ' cells with that of 4 x 10^6 Sca-1 ' cells, and also account for host BM cells surviving the irradiation. Both 200 Thy-1.1 'Lin-' Sca-1 ' cells and 4 x 10^6 Sca-1 ' cells would be expected to be contained in ~4 x 10^6 BM cells. The kinetics of recovery of WBCs, platelets, and hematocrit reconstitution with 200 Thy-1.1 'Lin-' Sca-1 ' cells or 4 x 10^6 whole BM cells, or the 200 Thy-1.1 'Lin-' Sca-1 ' cells + 4 x 10^6 Sca-1 ' BM cells are shown in Fig 8. As expected from Fig 6, the early recovery of WBCs is similar when either 200 Thy-1.1 'Lin-' Sca-1 ' cells or 4 x 10^6 whole BM cells are injected, but by day 28, whole
BM cell injected hosts had significantly more WBCs. The addition of $4 \times 10^5$ Sca-1 BM cells to 200 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells led to both 14-day and 28-day recoveries of WBCs that matched $4 \times 10^5$ whole BM cells. The addition of $4 \times 10^5$ Sca-1<sup>−</sup> BM cells to 200 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells also prevented the day 10 to 14 nadir in hematocrit seen with reconstitution with 200 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells alone, but added little to the small platelet reconstitution differences between 200 Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> cells injected and $4 \times 10^5$ whole BM injected hosts.

To identify directly the relative contribution of Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup> donor versus Sca-1<sup>−</sup> BM donor cells in the blood of reconstituted hosts, we used two congenic mouse strains (C57BL/Ka-Thy-1.1-Ly-5.1, C57BL/Ka-Thy-1.1-Ly-5.2 and their F1 [C57BL/Ka-Thy-1.2-Ly-5.1 x C57BL/Ka-Thy-1.1-Ly-5.2]). We stained with both anti-Ly-5.1 and anti-Ly-5.2 antibodies along with antibodies that define WBC subsets. Table 2 lists the cell types provided by each of the Ly-5 mouse strains. It would be expected that the addition of both Ly-5 alloantibodies to a mixture of BM cells from all three donor strains would give clean separation of the three cell populations, and thus allow quantitative analysis of the contribution of all three strains. However considerable overlap in the staining of Ly-5<sup>+</sup> Ly-5<sup>−</sup> cells with that of Ly-5<sup>−</sup> Ly-5<sup>+</sup> cells required setting analysis gates that included an area of overlap (Fig 9, panel 4 of Mac-1/Gr-1 or B220 population). Consequently, the data presented are semiquantitative. For example, in Fig 9, in the top row, the left-most panel FACS plot shows the staining of all Mac-1/Gr-1<sup>−</sup> cells and the gate set for positive cells (gray area). The second panel shows the staining of the host strain for Mac-1<sup>+</sup>/Gr-1<sup>−</sup> positive cells. The third panel shows donor A (Ly-5<sup>+</sup>) Gr-1/Mac-1<sup>−</sup> positive cells, and panel 4 shows donor B (Ly-5<sup>−</sup> Mac-1<sup>−</sup> Gr-1<sup>−</sup> positive cells).

Table 2. Strain Combinations Used to Compare Thy-1.1<sup>+</sup> Lin<sup>−</sup> Sca-1<sup>−</sup>, Sca-1<sup>−</sup>, and Radiosensitive Host Cells in a Competitive Repopulation Assay

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Cell Type Injected</th>
<th>Cell No. Injected</th>
<th>Staining With Antibodies</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/Ka-Ly-5.2</td>
<td>None</td>
<td>None</td>
<td>Ly-5.1&lt;sup&gt;+&lt;/sup&gt; Ly-5.2&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Host</td>
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<td>Thy-1.1&lt;sup&gt;+&lt;/sup&gt; Lin&lt;sup&gt;−&lt;/sup&gt; Sca-1&lt;sup&gt;−&lt;/sup&gt;</td>
<td>200</td>
<td>Ly-5.1&lt;sup&gt;+&lt;/sup&gt; Ly-5.2&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Donor A</td>
</tr>
<tr>
<td>(C57BL/Ka-Thy-1.2-Ly-5.1 x C57BL/Ka-Thy-1.1-Ly-5.2) F1</td>
<td>Sca-1&lt;sup&gt;−&lt;/sup&gt; or whole BM</td>
<td>$4 \times 10^5$</td>
<td>Ly-5.1&lt;sup&gt;+&lt;/sup&gt; Ly-5.2&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Donor B</td>
</tr>
</tbody>
</table>
cells. FACS analyses (the qualitative data) from samples from transplanted mice are shown in Figs 10 through 13, and a semiquantitative assessment of all samples analyzed is presented in Table 3.

FACS plots of the myelomonocytic cells from mice sacrificed at day 11 postirradiation and reconstitution are shown in Fig 10. The appearance of donor-derived myeloid cells is shown at day 11 (Fig 10), although some mice did not have detectable levels of myeloid cells. More evident results were obtained at day 14 postirradiation in the myelomonocytic and B cells (Fig 11A and B). The top row of Fig 11A shows 4 mice reconstituted with 200 Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cells from donor A strain only; all 4 panels show donor A Mac-1/Gr-1<sup>+</sup>-positive cells, but few or no host cells of this phenotype. The detection of signals in the donor B “box” gives a low, but detectable background, similar to Fig 10, row 1,
Fig 10. Comparison of hematolymphoid reconstitution of lethally irradiated mice 11 days after transplanting purified Thy-1.1^+ Lin^- Sca-1^+ cells, whole BM cells, or a combination of Thy-1.1^+ Lin^- Sca-1^+ cells and Sca-1^- cells. The recipient mice were transplanted with 200 Thy-1.1^+ Lin^- Sca-1^+ cells (donor A, first row), 4 \times 10^5 whole BM cells (donor B, second row), or the combination of 200 Thy-1.1^+ Lin^- Sca-1^+ (donor A) and 4 \times 10^5 Sca-1^- (donor B) cells (third row). See Fig 9 for gates set to select Mac-1/Gr-1^+ cells (panel 1), the Ly-5.1 versus Ly-5.2 profile for Mac-1/Gr-1^+ gated cells of the host strain (panel 2), donor A strain (panel 3), and donor B strain (panel 4). The PB cells of these mice were analyzed 11 days after transplantation. The percentages of Mac-1 and Gr-1^+ cells within donor A dominant and donor B dominant boxes are indicated in each panel.

The third row shows Gr-1/Mac-1^+ cells from hosts injected with 4 \times 10^5 unfractionated BM cells from donor B strain; all 3 panels show donor B but not host strain cells. Again, very considerable spillover is seen in the donor A box, and, collectively, these 4 panels plus panel 3, row 1 define the lower limit of donor A spillover into the donor B box that would be considered background. The bottom row of panels shows the results of 4 mice that were in the competitive repopulation experiment. All 4 panels show abundant reconstitution of Mac-1/Gr-1^+ cells from the injected 200 Thy-1.1^+ Lin^- Sca-1^+ cells from donor A strain, but no detectable or very few Mac-1/Gr-1^+ cells of donor B strain (4 \times 10^5 Sca-1^-) origin could be detected. No host reconstitution of Gr-1/Mac-1^+ cells was detectable. Similar results were obtained for the appearance of myeloid cells at day 11 (Fig 10), although some mice did not have detectable levels of myeloid cells. Thus, at days 11 and 14, 200 Thy-1.1^+ Lin^- Sca-1^+ cells are more active at providing Mac-1/Gr-1^+ cells than either 4 \times 10^5 Sca-1^- or endogenous host radioresistant cells.
Comparison of hematolymphoid reconstitution of lethally irradiated mice 14 days after transplanting purified Thy-1.1\(^*\) Lin\(^-\) Sca-1\(^+\) cells, whole BM cells, or a combination of Thy-1.1\(^*\) Lin\(^-\) Sca-1\(^+\) and Sca-1\(^-\) cells. The recipient mice were transplanted with 200 Thy-1.1\(^*\) Lin\(^-\) Sca-1\(^+\) cells (donor A, first row), 4 \(\times\) 10\(^5\) whole BM cells (donor B, second row), or the combination of 200 Thy-1.1\(^*\) Lin\(^-\) Sca-1\(^+\) (donor A) and 4 \(\times\) 10\(^5\) Sca-1\(^-\) (donor B) cells (third row). See Fig 9 for gates set to select Mac/Gr-1-positive cells (panel 1), the Ly-5.1 versus Ly-5.2 profile for Mac1/Gr1 gated cells of the host strain (panel 2), donor A strain (panel 3), and donor B strain (panel 4). The PB cells of these mice were analyzed 14 days after transplantation. The percentages of Mac1 and Gr-1\(^*\) cells (A) and B220\(^*\) (B) within donor A dominant and donor B dominant boxes are indicated in each panel.

At the same time point, as shown in Fig 11B, 200 Thy-1.1\(^*\) Lin\(^-\) Sca-1\(^+\) cells injected alone did not yet give rise to B cells (but see Fig 12B, 13B, and Table 3 for later time points), whereas 4 \(\times\) 10\(^5\) unfractionated BM cells did give rise to abundant numbers of B cells. At 14 days, 4 \(\times\) 10\(^5\) Sca-1\(^-\) cells were superior to 200 Thy-1.1\(^*\) Lin\(^-\) Sca-1\(^+\) cells in giving rise to PB B cells (bottom panel), although in this circumstance a few B cells were derived from Thy-1.1\(^*\) Lin\(^-\) Sca-1\(^+\) cells. Endogenous host cells did not contribute.

A sample of the results at 21 days postirradiation and injection is shown in Fig 12. In Fig 12A, 200 Thy-1.1\(^*\) Lin\(^-\) Sca-1\(^+\) cells injected alone (top row) or along with 4 \(\times\) 10\(^5\) Sca-1\(^-\) cells (third row) gave rise to the majority of signals from Gr-1/Mac-1-positive cells. Sca-1\(^-\) cells (4 \(\times\) 10\(^5\)) gave rise to low frequencies of Mac-1/Gr-1-positive cells, barely above background, but the host did not. When B-lineage cells were examined (Fig 12B), 200 Thy-1.1\(^*\) Lin\(^-\) Sca-1\(^+\) cells alone or in competition gave rise to abundant numbers of cells. Sca-1\(^-\) (4 \(\times\) 10\(^5\)) cells also contribute to B-lineage cells seen at 21 days, although their relative contribution...
wanes in comparison to that at day 14. Similar results for Gr-1/Mac-1 and B220+ cells are seen at day 29 (Fig 13A and B). In Fig 13C, in contrast to B and myelomonocytic cells, endogenous CD3+ T cells are easily detectable and are probably derived from radioresistant T cells derived from 200 Thy-1.1b Lin- Sca-1+ cells alone or in competition are a major source of Gr-1/Mac-1-positive cells at all assay intervals (10, 14, 21, and 29 days) and contribute significantly to B cells on day 21 and thereafter. Unfractionated BM cells (4 × 10^5) give rise to both Gr-1/Mac-1- and B220-positive cells at the earliest time they can be detected. Sca-1- cells (4 × 10^5) are poor myelomonocytic progenitors, but contain significant but short-term B-cell reconstitutive activity.

Within the Thy-1.1b Lin- Sca-1+ population, the Rh123+ subset preferentially contributes to the early appearance of myelomonocytic progeny. The long-term repopulation capacity of whole BM or of enriched HSC-containing subsets

The semiquantitative assessment of PB Gr-1/Mac-1- and B220-positive cells and their donor progenitor sources are presented in Table 3. Two hundred Thy-1.1b Lin- Sca-1+ cells alone or in competition are a major source of Gr-1/Mac-1-positive cells at all assay intervals (10, 14, 21, and 29 days) and contribute significantly to B cells on day 21 and thereafter. Unfractionated BM cells (4 × 10^5) give rise to both Gr-1/Mac-1- and B220-positive cells at the earliest time they can be detected. Sca-1- cells (4 × 10^5) are poor myelomonocytic progenitors, but contain significant but short-term B-cell reconstitutive activity.
Fig 12. Comparison of hematolymphoid reconstitution of lethally irradiated mice 21 days after transplanting purified Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} cells, whole BM cells, or a combination of Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} cells and Sca-1\textsuperscript{−} cells. The recipient mice were transplanted with 200 Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} cells (donor A, first row), 4 x 10\textsuperscript{5} whole BM cells (donor B, second row), or the combination of 200 Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} (donor A) and 4 x 10\textsuperscript{5} Sca-1\textsuperscript{−} (donor B) cells (third row). See Fig 9 for gates set to select Mac/Gr-1-positive cells (panel 1), the Ly-5.1 versus Ly-5.2 profile for Mac-1/Gr-1 gated cells of the host strain (panel 2), donor A strain (panel 3), and donor B strain (panel 4). The PB cells of these mice were analyzed 21 days after transplantation. The percentages of Mac-1 and Gr-1\textsuperscript{+} cells (A) and B220\textsuperscript{−} (B) within donor A dominant and donor B dominant boxes are indicated in each panel. During the FACS analysis, one of the Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} cell samples was contaminated with donor B cells as indicated by the arrow (row 1, first panel). The samples for the combination of 200 Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} (donor A) and 4 x 10\textsuperscript{5} Sca-1\textsuperscript{−} (donor B) cells were always analyzed after those from the 200 Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} group (donor A only).

appears to be concentrated in the 2% to 5% of BM cells that largely exclude Rh123 from mitochondrial staining (Rh123\textsuperscript{−}),\textsuperscript{1,3} whereas Rh123\textsuperscript{+} cells appear to be more enriched in short-term but not in long-term in vivo double transfer reconstitution type assays (pre-CFU-S assay).\textsuperscript{22} To assess directly whether the Gr-1/Mac-1 reconstitution patterns seen with Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} cells is also divisible along these lines, the congenic mouse lines were used for competitive reconstitution using 50 Rh123\textsuperscript{+} versus 200 Rh123\textsuperscript{−} Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} cells, roughly their proportions in the Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} pool. The results, shown in Fig 14, demonstrated that at days 10 to 14, 200 Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} Rh123\textsuperscript{−} cells can give rise to donor-derived Gr-1/Mac-1 blood cells significantly better ($P < .002$) than the Rh123\textsuperscript{+} subset. However, by days 21 to 28, 50 Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} Rh123\textsuperscript{+} cells give equivalent or slightly better reconstitution than 200 Thy-1.1\textsuperscript{hi} Lin\textsuperscript{−} Sca-1\textsuperscript{−} Rh123\textsuperscript{−} cells (see Fig 14 legend).
**Cellularity of BM.** To identify early foci of hematopoiesis generated by Thy-1.1\textsuperscript{lo} Lin\textsuperscript{-} Sca-1\textsuperscript{+} HSCs, BM sections were evaluated (Fig 15) 7 days after irradiation/reconstitution. When the irradiated mice did not receive cells, we did not observe any foci of hematopoiesis throughout the BM sections (Fig 15A). After the injection of 100, 500, or 1,000 Thy-1.1\textsuperscript{lo} Lin\textsuperscript{-} Sca-1\textsuperscript{+} cells, distinct foci of hematopoiesis in the bone marrow could be detected at 7 days, mainly in proximity to the endosteum (Fig 15B). These foci were predominantly myeloid (Fig 13C), whereas some "pure" megakaryocytic colonies were also found, usually at some distance from the endosteum (Fig 15D).

**DISCUSSION**

Although it is clear that the critical cell population for long-term repopulation of the hematolymphoid system in BMT are HSCs, it has been reasonable to assume (and in fact is part of the conventional wisdom) that other hematopoietic progenitors, and even immediate precursors of blood cells, are the major contributors to the early phases of hematopoietic recovery. Until progenitor and more mature cells could be separated from HSCs, it was impossible to test this conventional wisdom, and therefore impossible to determine the extent to which stem cells contribute to early hematopoietic recovery as well as long-term marrow reconstitution. We have defined HSCs as cells that are each capable of differentiation to all blood cells and elements (multilineage differentiation) and self-renewal. Thy-1.1\textsuperscript{lo} Lin\textsuperscript{-} Sca-1\textsuperscript{+} cells (but no other cells in C57BL/Ka-Thy1.1 BM) are highly enriched for and perhaps entirely composed of HSCs. Thy-1.1\textsuperscript{lo} Lin\textsuperscript{-} Sca-1\textsuperscript{+} cells are biologically heterogeneous at the single-cell level; one example is how long each clone sustains...
hematopoiesis (and presumably self-renewal) in vivo\textsuperscript{[23,36,37]} (Uchida and Weissman, manuscript in preparation, and Morrison and Weissman, manuscript in preparation). Myelomonocytic cells (Mac-1/Gr-1\textsuperscript{+}) are short-lived in vivo. B and T cells can undergo extensive amplification and generation of long-term memory cells in response to antigenic stimulation. Therefore, we believe that the assessment of Mac-1/Gr-1\textsuperscript{+} cells in the blood is most sensitive for the early and sustained activity of progenitors. In this report, we present evidence that the early phase (7 to 21 days) and middle phase (21 to 35 days) of hematopoietic recovery in lethally irradiated mice can be achieved exclusively through the proliferation and differentiation of purified Thy-1.1\textsuperscript{+} Lin\textsuperscript{-} Sca-1\textsuperscript{-} HSCs. The minimum number of Thy-1.1\textsuperscript{+} Lin\textsuperscript{-} Sca-1\textsuperscript{-} stem cells required to radioprotect 95\% to 100\% of animals (PD\textsubscript{50}) is 100 cells.\textsuperscript{[18]} One hundred to 200 Thy-1.1\textsuperscript{+} Lin\textsuperscript{-} Sca-1\textsuperscript{-} cells give rise to donor-derived B cells, myeloid cells, platelets, and RBCs within 2 to 3
weeks. Increasing the dose of Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> stem cells to 500 cells led to a more rapid recovery of all cell types tested, but provision of 1,000 such cells was not significantly more proficient in a quantitative or kinetic sense for the production of WBCs and RBCs (Fig 3). Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cells represent about 0.05% of whole BM, and therefore 1 x 10<sup>6</sup> BM cells should contain on the order of 500 Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cells. When 1 x 10<sup>6</sup> BM cells were compared with 500 Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cells in lethally irradiated mice, there was only a minor delay in the appearance of platelets and increase in hematocrit (1 to 3 days) at day 14 when HSCs are used, and no kinetic difference for the early increase of WBCs (mainly myelomonocytic cells), although in these experiments BM cells eventually gave rise in the mid-term (days 21 to 28 in 2 of 3 experiments) to higher WBC counts than their stem cell equivalents. When 200 Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cells were compared with 4 x 10<sup>5</sup> whole BM cells, again no significant kinetic difference in the recovery of donor-derived myelomonocytic cells was observed.

After the initial submission of this report, a study of hematopoietic reconstitution of 13 Gy (650 R x 2) irradiated hosts with Lin<sup>-</sup> Sca-1<sup>+</sup> BM cells was published by Bradford et al., showing a very significant and specific delay in the appearance of platelets compared with WBCs and RBCs in the blood. This thrombocytopenia worsened when reconstituted mice were challenged with sublethal doses of 5-FU or cyclophosphamide. These results of Bradford et al clearly differ from the results reported here, although Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> cells certainly would be included in the Lin<sup>-</sup> Sca-

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**Figure 13. (Cont'd)**

B

**B220<sup>+</sup> gated**

200 Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> Cells (Donor A only), n=4

4 x 10<sup>5</sup> BM Cells (Donor B only), n=2

200 Thy-1.1<sup>+</sup> Lin<sup>-</sup> Sca-1<sup>+</sup> (Donor A) & 4 x 10<sup>5</sup> Sca-1<sup>-</sup> Cells (Donor B), n=3
CD3 \(^+\) gated

200 Thy-1.1\(^{10}\) Lin\(^-\) Sca-1\(^+\) Cells (Donor A only), n=4

4 \(\times 10^5\) BM Cells (Donor B only), n=2

200 Thy-1.1\(^{10}\) Lin\(^-\) Sca-1\(^+\) (Donor A) \& 4 \(\times 10^5\) Sca-1\(^-\) Cells (Donor B), n=3

Fig 13. (Cont’d)

1\(^+\) population injected by Bradford et al.\(^{38}\) Unfortunately, Bradford et al.\(^{39}\) did not include a comparison of 13 Gy irradiated mice injected with unfractonated BM, and so it is impossible to determine whether the thrombocytopenia observed resulted from a defect in the Lin\(^-\) Sca-1\(^+\) population, the high-dose irradiation protocol (650 R \(\times\) 2 or 425 R \(\times\) 2 protocols used here), or both. The doses of irradiation used in the experiments described here were fully lethal and excluded host nucleated blood reconstitution over the period assayed, except for T cells. At the time of our experiments, a dose of 650 R \(\times\) 2 led to death by a radiation syndrome that could not be protected by BM cell injection. The Bradford et al.\(^{39}\) study could point to a potential serious problem with supralethal doses of irradiation, and it will be of interest to learn whether the thrombocytopenia they observe is present in mice receiving doses of unfractonated BM cells that contain \(\sim\)1,000 Lin\(^-\) Sca-1\(^+\) BM cells. If so, perhaps host elements critical for thrombopoiesis are selectively radiosensitive in the 8.5 to 13.0 Gy (850 to 1,300 R) range.

In contrast to Thy-1.1\(^{11}\) Lin\(^-\) Sca-1\(^+\) HSCs, the \(\sim\)96% of BM that is Sca-1\(^-\) lacks significant progenitors, as measured by generation of myelomonocytic cells. However, Sca-1\(^-\) cells do contain short-term B-lineage progenitors. Taken together, these observations quite clearly indicate a major role for stem cells (as a population) in both early and late hematopoietic recovery, and do not provide strong evidence for a major role by committed progenitors in the early phases of hematopoietic recovery. These observations are consistent with data from a previous study in which irradiated mice
Fig 14. Competitive reconstitution assay for hematolymphoid recovery of lethally irradiated mice achieved by combination of purified Rh123lo and Rh123hi subsets of Thy-1.1lo Lin- Sca-1+ cell. The recipient mice were transplanted with the combination of 50 Rh123lo Thy-1.1lo Lin- Sca-1+ cells (donor A) and 200 Rh123hi Thy-1.1lo Lin- Sca-1+ cells (donor B), except where indicated. See Fig 9 for gates set to select Mac-1/Gr-1+ gated cells (panel 1), the Ly-5.1 versus Ly-5.2 profile for Mac-1/Gr-1 gated cells of the host strain (panel 2), donor A strain (panel 3), and donor B strain (panel 4). The top row shows Mac-1/Gr-1 versus Ly-5.1 staining of the PB sample 10 to 11 days after the transplantation. The PB analyses of Mac-1/Gr-1+ cells at day 10 to 11 (A) and day 28 (B) are shown. Note that the Rh123lo cells came from donor A strain in row 3, and from donor B strain in row 4; and that the Rh123hi cells came from donor B strain in row 3 and from donor A strain in row 4. In this particular experiment, all recipient mice were infected with Pasteurella pneumotropica before irradiation, as determined by an overgrowth of Pasteurella pneumotropica from nasopharynx of mice that died ≤10 days after transplantation. Even surviving mice had reduced levels of donor cell engraftment evident by the analysis at day 28. Relatively high levels of endogenous Mac-1/Gr-1+ positive cells were detected as a result (see Fig 13A for a comparison of the same time point). In this experiment, donor B F1 cells were Thy-1.1 homozygous.
Table 3. Competitive Reconstitution of PB Mac-1/Gr-1^-Positive Cells by 200 Thy-1.1^+ Lin^- Sca-1^- Versus 4 × 10^5 Sca-1^- BM Cells

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<td>High</td>
<td>8</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>(\chi^2)</td>
<td>8.4</td>
<td>12</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>(P)</td>
<td>&lt;.005</td>
<td>&lt;.005</td>
<td>&lt;.005</td>
<td>&lt;.01</td>
</tr>
</tbody>
</table>

PBS samples from transplanted animals were harvested and processed as described in the Materials and Methods. Cells were stained for antibodies against Ly-5.1, Ly-5.2, and Mac-1/Gr-1, and FACS results were shown in Fig 9 (day 11), Fig 10A (day 14), Fig 11A (day 21), and Fig 12A (day 28). To confirm low levels of Sca-1^- derived (donor B) Mac-1/Gr-1^- cells, Ly-5.1^+ (either donor) cells were plotted against Mac-1/Gr-1 and Ly-5.2 in logarithmic plots. For example, in Fig 9, the 5 donor A plots (row 1, panel 2; row 2, all 4 panels) show 0.4% to 2.1% of the signals from Ly-5.1 only cells in the Ly-5.2 "box." Two samples in row 1 (panels 2 and 4) fell within that range and were designated negative, and two (panels 1 and 3) were above that range but clearly quite different from the Ly-5.1^+ Ly-5.2^- "box," and were designated positive. Only an evident Ly-5.1^+ Ly-5.2^- and Mac-1/Gr-1^- population was scored as a positive.

received 2 × 10^5 non-HSCs, either Thy-1.1^-, Sca-1^-, or Lin^- BM cells; together, these cells represent 99.95% of BM cells, yet irradiated hosts receiving them survived ≥6 days longer than mice receiving no cells. In that study, both 4 × 10^5 Sca-1^- and Lin^- BM cells contain relatively short-lived B-lineage progenitors, whereas 2 × 10^5 Thy-1.1^- cells included progenitors for B-lineage cells for at least 14 weeks. The relatively short lifespan of B-lineage cells derived from Sca-1^- progenitors may make them valuable to develop in vivo assays to study the effect of antigens and/or other agents on selection, outgrowth, and longevity of cells of the B lineage.

The enhanced production of WBCs and, to some extent, platelets at 21 and 28 days postreconstitution in mice receiving 1 × 10^5 BM cells as compared with 500 Thy-1.1^+ Lin^- Sca-1^- cells in two of three experiments could deserve further investigation. Perhaps another class of cells in the BM is involved in this second phase of recovery, e.g., BM T cells producing hematopoietic cytokines; or perhaps engraftment of more lineage-committed progenitors results in a 14- to 21-day lag before their contribution to WBCs is apparent.

Spangrude and Johnson showed that Thy-1.1^+ Lin^- Sca-1^- cells can be further subdivided into rhodamine-123 low and high (Rh-123^hi, Rh-123^lo) subsets. Rh-123^hi Thy-1.1^+ Lin^- Sca-1^- cells were highly enriched for day-13 CFU-S activity, but were not correspondingly enriched for pre-CFU-S or hematopoietic repopulation activity when the experiments were performed. We show here that the early phase of hematopoietic engraftment is mainly achieved by the Rh-123^lo Thy-1.1^+ Lin^- Sca-1^- subset, whereas the Rh-123^lo Thy-1.1^+ Lin^- Sca-1^- subset contributes more substantially to mid-phase multilineage reconstitution. Moreover, the compartment of Thy-1.1^+ Lin^- Sca-1^- cells are clearly heterogeneous in terms of repopulation kinetics and sustained hematopoietic potential, a well as for a number of other parameters. Elsewhere, we report that phenotypic markers can define within the Thy-1.1^- Lin^- Sca-1^- population subsets highly enriched for early versus mid versus sustained hematopoiesis (Morrison et al, manuscript in preparation). The biologic bases for these differences are not yet known, nor is the lineage relationship between these subsets.

That is not to say that hematopoietic progenitors cannot also contribute to early and late hematopoiesis. Thy-1.1^- cells, Lin^- cells, and Sca-1^- cells can all contribute substantially to B lymphopoiesis. Still, it is likely that the most important blood elements in the early phases of hematopoietic recovery are myeloid, and/or platelets, and/or erythrocytes to protect the hosts from infection, bleeding, and anemia, respectively. Perhaps the repopulation ability of non-HSC progenitor cells in vivo may not be sufficient to contribute significant levels of blood elements during the early phases of hematopoietic recovery, because they can only undergo a limited number of cell divisions; or perhaps they are less efficient at seeding hematopoietic microenvironments than HSCs.

In these studies, the majority of early and late hematopoiesis that is donor-derived after BMT appears to be caused by the HSC content of the BM inoculum. This finding raises several questions. For example, do the HSCs home preferentially to BM and land in self-renewal microenvironments in...
a BMT situation, in contrast to other committed hematolymphoid progenitors? Or is it likely that committed progenitors have a sufficiently limited number of cell divisions they can undergo that they simply cannot provide the major outcome of hematopoiesis that HSCs provide? Another possibility is that the frequency of HSCs that enter the proliferative and differentiative cycle is dependent on the frequency and state of differentiation of other non-stem cells in the BM or the blood, so that the provision of large numbers of hematopoietic progenitors suppresses early cell divisions by hematopoietic stem cells, even in the lethally irradiated host. It shall be interesting to determine the fraction of HSCs that enter the mitotic cycle in lethally irradiated animals in the presence or absence of added hematopoietic progenitors and more mature BM cells.

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Rapid and sustained hematopoietic recovery in lethally irradiated mice transplanted with purified Thy-1.1lo Lin-Sca-1+ hematopoietic stem cells

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