Thymic Stem Cells in Mouse Bone Marrow

By M. Antica, L. Wu, K. Shortman, and R. Scollay

There is still controversy concerning the nature of the stem cells from bone marrow that colonize the thymus during embryogenesis and continually throughout life. To identify the bone marrow stem cells that home to and populate the thymus, we screened murine bone marrow cells for the presence of a population of surface phenotype similar to the earliest known intrathymic precursor. We have identified a population characterized by expression of an intermediate level of heat stable antigen, a very low level of Thy-1, and high levels of CD44 and class I major histocompatibility complex antigens. It is negative for B-cell, granulocyte, macrophage, and erythrocyte markers (B220, Gr-1, Mac-1, and TER-119). All these markers are common to the intrathymic precursors and bone marrow stem cells. However, this new bone marrow population is Sca-2+, similar to the intrathymic precursor, which makes a clear distinction from the Sca-2− bone marrow hematopoietic stem cells previously characterized.

All the cells of the hematolymphoid system develop from a common precursor known as the multipotent hematopoietic stem cell. This cell resides in the liver during fetal development and in the bone marrow in the adult. At some point during the development, commitment to one particular lineage occurs, with the loss of ability to give rise to other lineages. It has been proposed that, for the lymphocyte lineages, there exists an intermediate stage that gives rise to both B and T lymphocytes, but not to other lineages. So far, this intermediate committed lymphoid precursor has not been convincingly demonstrated in bone marrow. On the other hand, recent studies on the adult mouse thymus showed the existence of a population of precursor cells that had lost the ability to develop into myeloid or erythrocyte cells, but retained the ability to develop into T, B, and dendritic cells. This intrathymic population, representing 0.05% of all thymocytes, precedes in development the more numerous and better known CD4+CD8+ precursor cells. It has T-cell receptor (TCR) and Ig genes in germline state. On intrathymic transfer, it gives rise to T cells (both αβ and γδ) and to dendritic cells, but not to other lineages; on intravenous transfer, it also gives rise to B cells, but not to cells of the erythroid or myeloid lineages. This intrathymic precursor population lacks most T-cell antigens (CD2, CD3, CD8, and interleukin-2 receptor [IL-2R]), but expresses low levels of Thy-1 and moderate levels of CD4, and is therefore termed the "low CD4 precursor." It expresses moderate levels of heat stable antigen (HSA) and high levels of H-2K and CD44 (Pep-1). It does not express antigens characteristic of myeloid (Mac-1 and Gr-1), erythroid (TER-119), or B-cell (B220) lineages.

This low CD44 precursor population of cells is similar in surface phenotype to the previously described multipotent hematopoietic stem cell from the bone marrow, except for the expression of Sca-2 antigen, a marker found on the intrathymic low CD44 precursors but not on the hematopoietic stem cells in the bone marrow. Despite this detailed knowledge of the bone marrow multipotent stem cell and the intrathymic "lymphoid committed" precursor cell, the identity of the cell that actually migrates to and populates the thymus is still unknown.

Multipotent stem cells have been difficult to detect in the thymus, suggesting that some restriction in developmental potential occurs before or immediately after seeding the thymus. In an attempt to clarify this issue, we have looked in the bone marrow for a population of cells resembling in surface antigenic phenotype the thymic low CD4 precursor population. In this report, we describe a bone marrow population that appears to be intermediate between the multipotent stem cell and the earliest intrathymic precursor.

Materials and Methods

Mice. C57BL/Ka Thy-1.1, C57BL/6-Ly 5.1-Pep1, CBA, and BALB/c mice were bred and maintained in the Walter and Eliza Hall Institute Animal Facility. For intrathymic and intravenous lineage reconstitution, 4 to 6-week-old C57BL/Ka Thy-1.1 (Thy 1.1 and Ly 5.2) mice were used as donors and C57BL/6-Ly 5.1-Pep1 (Thy-1.2 and Ly 5.1) mice at 8 to 12 weeks of age were used as recipients. Cell suspensions. Mice were killed with CO2 and their organs were removed immediately into cold mouse tonicity buffered balanced salt solution (BSS) containing 5% fetal calf serum. Monoclonal antibodies (MoAbs) and reagents. The antibodies used for magnetic bead depletion were: anti-B220, clone RA3-6B2'; anti-Mac-1, clone M1/70.15*; anti-Gr-1, clone RB6-8C5*; and anti-erythrocyte antigen, clone TER-119 (kindly provided by Tatsuo Kina).
cells were excluded from the analysis in the same channel. The three parameters to be recorded for each analyzed cell. For analysis, 25 Gy y-irradiation with a 3-hour interval between). Antibiotics were added to the drinking water. On day 12, the spleens were removed. PepSh) were irradiated (7.5 Gy y-irradiation) in detail. The Ly 5.1 recipient mice were lethally irradiated (two doses of 5.5 Gy y-irradiation with a 3-hour interval between) 1 to 4 hours before IV transfer. The cells from Ly 5.2 mice were injected IV, along with 4 X 10^7 Ly 5.1 (recipient type) unfractionated bone marrow cells, to ensure long-term survival of the recipients. Antibiotics were added to the drinking water. At various times after transfer, the spleen and lymph nodes (pooled), thymus, and bone marrow were collected from recipients. Cells were stained in two colours with donor-specific anti-Ly 5.2 (FITC-conjugated) together with biotinylated lineage-specific antibodies, followed by PE-avidin for the second stage, as described above. In some experiments, where noted, three-color staining was used for analysis.

RESULTS

Enrichment of lineage-negative cells from mouse bone marrow. The bone marrow contains a large variety of hematopoietic cells ranging from multipotent stem cells to fully differentiated cells. To enrich precursor cells, the bone marrow cell suspension was first depleted (immunomagnetic bead depletion) of the more mature stages of different lineages with antibodies to lineage-specific differentiation antigens (Lin). The antibodies were as follows: for B cells, anti-B220; for myeloid cells, anti-Gr-1 and anti-Mac-1; and, for erythroid cells, TER-119. The efficiency of the depletion was monitored by staining with a fluorochrome-labeled anti-rat Ig antibody. Figure 1A shows the staining before and after depletion of the Lin" cells. This depletion procedure removed most of the Lin" cells, and the Lin" cells could be selected by software gating from the residual Lin" cells. Cells were considered Lin" if they fell under the curve of the negative control (Fig 1A). The proportion of Lin" cells in the depleted preparation was consistently about 20% and represented approximately 80% of the original population.

Characterization of the Lin" cells by surface marker analysis. To screen for the presence of the low CD4 precursors or their immediate progenitors in the Lin" bone marrow, four-color flow cytometric analysis was performed. Among the Lin" bone marrow cells, a population expressing the low levels of Thy-1 and intermediate levels of HSA was selected and sorted on the basis of Sca-2 expression. Reanalysis showed that the purity of the sorted populations was greater than 97%.

Spleen colony assay (colony-forming unit spleen [CFU-S]). The spleen colony assay of Till and McCulloch was applied. Sorted bone marrow cells or thymic low CD4 precursor cells were injected intravenously (IV) into lethally irradiated mice (two doses of 5.5 Gy y-irradiation with a 3-hour interval between). Antibiotics were added to the drinking water. On day 12, the spleens were removed and fixed in Bouin's solution, and macroscopically visible colonies were counted.

Thymic reconstitution assay. The procedure has been described in detail. The Ly 5 congenic recipient mice (C57BL/6-Ly-5.1-Pep5^3) were irradiated (7.5 Gy y-irradiation) 1 to 3 hours before intrathymic transfer. The cells in 10 ^3 of BSS were injected directly into a single lobe of the recipient thymus. At various times after transfer, cell suspensions were prepared from the injected recipient thymus lobes and stained with FITC-conjugated antibody against the donor-type Ly 5, together with either anti-Thy-1, anti-B220, anti-Gr-1, or anti-Mac-1 (biotinylated, with PE-Av second stage). The stained cells were then analyzed by flow cytometry, with gating for Ly 5 donor-derived cells. List mode files 5 to 10 X 10^6 events were collected for determination of the percentage of donor-derived cells.

Lineage reconstitution assay. The procedure was similar to that described by Spangrude and Scollay. The Ly 5.1 recipient mice were lethally irradiated (two doses of 5.5 Gy y-irradiation with a 3-hour interval between) 1 to 4 hours before IV transfer. The cells from Ly 5.2 mice were injected IV, along with 4 X 10^7 Ly 5.1 (recipient type) unfractionated bone marrow cells, to ensure long-term survival of the recipients. Antibiotics were added to the drinking water. At various times after transfer, the spleen and lymph nodes (pooled), thymus, and bone marrow were collected from recipients. Cells were stained in two colours with donor-specific anti-Ly 5.2 (FITC-conjugated) together with biotinylated lineage-specific antibodies, followed by PE-avidin for the second stage, as described above. In some experiments, where noted, three-color staining was used for analysis.
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Fig 1. Enrichment and analysis of bone marrow cells. (A) Bone marrow staining with Lin markers (B220, Gr-1, Mac-1, and TER-119) before (upper panel) and after (lower panel) depletion with anti-lg magnetic beads. (B) HSA and Thy-1 expression on Lin-depleted bone marrow cells gated for Lin'. (C) Multiparameter analysis of Lin'Thy-1'HSA' cells. Shaded lines represent the level of staining with isotype-matched irrelevant antibodies. The top box represents forward scatter (FS) and the axis is on a linear scale. The other boxes show log fluorescence for the antigens indicated in each box.

also some more mature cells, also was also expressed by the Lin'Thy-1'HSAl bone marrow cells (Fig 1C). We have previously shown that virtually all the intrathymic low CD4 precursors are c-kit, and all the reconstituting activity on intrathymic (IT) transfer into irradiated recipients was retained in the c-kit fraction. Although c-kit and all the antigens described above are characteristic of the thymic low CD4 precursors, they are also diagnostic for the bone marrow multipotent stem cells. The only marker known to be expressed on the thymic low CD4 precursors but not on the bone marrow multipotent stem cells is the stem cell antigen 2 (Sca-2). This antigen was found on 30% of undepleted bone marrow cells (Fig 2), as shown elsewhere. It was expressed by B220' cells but also by 12% of other bone marrow cells that were B220' and of larger size (Fig 2). Among the Lin'Thy-1'HSAl bone marrow cells, about 60% were Sca-2' (Fig 1C). Sca-2 therefore represented a marker that might discriminate thymic stem cells from the bone marrow multipotent stem cells.

When stained for CD4, the Lin'Thy-1'HSAl fraction of

Fig 2. Expression of Sca-2 and B220 antigens on bone marrow cells.
bone marrow showed only a marginal shift of fluorescence above background (Fig 1C). This was substantially lower staining than that given by the intrathymic low CD4 precursors stained under the same conditions. Because the Sca-2+ cells are included in this analysis, it follows that the Sca-2+ precursors are also CD4- or very low.

Because there are conflicting reports on CD4 expression on bone marrow cells, a multiparameter analysis of undepleted bone marrow cells from various mouse strains was performed. The undepleted bone marrow cells were stained with the anti-CD4 antibody together with T-cell (anti-Thy-1), B-cell (anti-B220), myeloid (anti-Gr-1 and anti-Mac-1) and erythroid markers (TER-119). Bone marrow included a proportion of cells that stain with anti-CD4 and this proportion varies slightly (3% to 4%) with mouse strain (Fig 3). About half of these express both high levels of CD4 and high levels of Thy-1, suggesting that they are mature CD4+ T cells in the bone marrow. When the remaining CD4+Thy-1−Lin− cells were selected by electronic gating (rectangle in Fig 3A) and analyzed for B-cell and myeloid marker expression, they were found to express at least one of the lineage markers. Most CD4-bearing cells in the bone marrow are therefore not stem cells.

Hematopoietic reconstitution potential of the sorted Sca-2+ bone marrow cells. The Lin− bone marrow cells were stained with anti-Thy-1, anti-HSA, and anti-Sca-2 antibodies, and the Lin−Thy-1−HSA−Sca-2− cells (further referred to in text as Sca-2+) were selected and sorted for functional analysis.

To test the T-precursor potential of the sorted Sca-2+ cells, they were injected into the thymuses of sublethally irradiated animals. Table 1 shows that this Sca-2+ population reconstituted an irradiated thymus and produced double-positive (CD4+CD8+) and single-positive (CD4+CD8− and CD4+CD8−) thymocytes. From the ratio of the absolute number of progeny to the number of injected Sca-2+ cells,
PROTHYMOCYTES IN BONE MARROW

Table 1. Thymic Reconstitution 3 Weeks After IT Transfer of Bone Marrow and Thymic Precursors

<table>
<thead>
<tr>
<th>Donor-Derived Subpopulations (%)</th>
<th>Cells Transferred</th>
<th>No. of Cells Recovered (x10^5)</th>
<th>Ratio of Recovered/Injected*</th>
<th>CD4^8</th>
<th>CD4^8</th>
<th>CD4^8</th>
<th>CD4^8</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCa-2^+</td>
<td>10^2</td>
<td>10.8 ± 10.7</td>
<td>10 ± 4</td>
<td>86 ± 4</td>
<td>2.4 ± 0.8</td>
<td>1.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Low CD4 precursors</td>
<td>10^4</td>
<td>7.6 ± 2</td>
<td>8 ± 10^3</td>
<td>0.2 ± 0.1</td>
<td>90 ± 5</td>
<td>7 ± 1.2</td>
<td>2.8 ± 1.1</td>
</tr>
</tbody>
</table>

The SCa-2^+ bone marrow precursors (Lin^-Thy-1^+HSA^+SCa-2^+) were sorted for Lin^- bone marrow as in Fig 1. The intrathymic low CD4 precursors were isolated as previously described. Both precursors were transferred into the thymus of irradiated mice differing at the Thy-1 locus. Three weeks later, the recipient thymuses were analyzed for donor-derived cells as described in Materials and Methods. Results summarized in this table are the means ± SD of three experiments each involving a pool of three recipient mice.

* Ratio determined by dividing the absolute number of donor cells recovered from the thymus at 3 weeks after injection by the number of cells originally injected intrathymically.

† Lin-Thy-1^+HSA^+SCa-2^+ bone marrow precursors.

Table 2. CFU-S Activity of Bone Marrow or Thymic Precursors

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of Cells Transferred</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow hematopoietic stem cells*</td>
<td>10^3</td>
<td>&gt;20†</td>
<td>All &gt; 20</td>
</tr>
<tr>
<td>Bone marrow SCa-2^+</td>
<td>2 × 10^3</td>
<td>11</td>
<td>7-17</td>
</tr>
<tr>
<td>Thymic low CD4 precursor cells</td>
<td>3 × 10^4</td>
<td>10</td>
<td>9-11</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow SCa-2^+</td>
<td>6 × 10^3</td>
<td>25</td>
<td>23-27</td>
</tr>
<tr>
<td></td>
<td>10^3</td>
<td>7</td>
<td>5-9</td>
</tr>
<tr>
<td></td>
<td>10^4</td>
<td>1</td>
<td>0-1</td>
</tr>
</tbody>
</table>

Lin indicates expression of the hemolymphoid lineage markers Mac-1, Gr-1, B220, and TEB-119 by the sorted fraction of cells. The full details of the spleen colony assay are given in Materials and Methods. Values for CFU-S numbers are the mean and range of three to five individual recipients. Two separate experiments are shown.

* Lin^-Thy-1^+HSA^-SCa-2^-.
† Confluent, unable to count.
‡ Lin-Thy-1^+HSA^+SCa-2^- bone marrow cells.

DISCUSSION

In this work, we describe a novel population of bone marrow precursor cells that was characterized with respect to expression of 15 cell surface markers as well as functional activity in vivo. This population appears to be phenotypically and functionally intermediate between the multipotent bone marrow stem cells described by several groups and the recently described intrathymic low CD4 precursor cells.

All three populations of precursor cells express low levels of Thy-1 antigen, are SCa-2^-, and are negative for differentiation markers for B cells, myeloid, and erythroid cells (Lin^-). All three populations express HSA, CD44, and H-2K antigens, and the stem cell factor receptor, c-kit. However these precursor populations can be distinguished by two other surface markers. Cells in this novel population are SCa-2^- like the intrathymic low CD4 precursors, but unlike the multipotent stem cells. They are CD4^- (or very low), like the multipotent stem cells, but unlike the low CD4 precursors.

The issue of CD4 expression on stem cells is somewhat confused. According to Fredrickson and Basch, all the pre-
cursor activity in the bone marrow is contained in the CD4+ fraction of cells. These investigators compared the activity of these CD4+ cells from a number of different mouse strains and concluded that the CD4+ fraction contains all the bone marrow stem cell activity. Because the bone marrow cells we describe here were almost negative for CD4, we analyzed the CD4 expression on undepleted bone marrow cells. A multiparameter analysis of bone marrow cells with anti-CD4 antibody together with lineage differentiation markers showed that most of the cells expressing CD4 were also positive for lineage differentiation markers. We showed in three different mouse strains that the CD4 high-positive cells were also Thy-1+, and that the remaining CD4 low-positive bone marrow cells expressed at least one other lineage marker, either B220, Mac-1, or Gr-1. The lineage-negative fraction of bone marrow cells did not express the CD4 antigen. A recent report suggests that all the precursor activity of bone marrow is contained in the fraction of bone marrow cells that becomes CD4+ only when labeled with a very high concentration of anti-CD4 antibodies (100 μg/mL). This report seems to disagree with the previous work of Fredrickson and Basch, who found all the precursor activity already in the fraction of bone marrow cells stained with standard concentrations of anti-CD4 antibodies (0.5 μg/mL). Because in both these studies the sorted CD4+ population of cells was not a distinct peak in the FACS profile (the peak appeared only if cells were analyzed in the FACScan machine, which has a higher resolution, but does not have the option of sorting the cells), it is possible that the cells described by Wineman et al were already included in the CD4+ population selected by Fredrickson and Basch. However, the Lin–Sca-2+ cells we describe here remained negative or extremely low for CD4 staining even when high concentrations of anti-CD4 antibody were applied. It is possible that this result was influenced by our staining procedure. However, after staining with anti-CD4 antibody under the conditions used to demonstrate CD4 expression on the intrathymic low CD4 precursors, the bone marrow Sca-2+ cells remained CD4+.

The bone marrow cells we describe here, Lin–Thy-1+HSA–Sca-2+, had T-cell precursor activity when they were tested in reconstitution assays. If they were transferred into thymuses of irradiated mice, they developed into T lymphocytes, as did the intrathymic low CD4 precursors, but their expansion potential was higher, ie, $2 \times 10^5$ compared with $10^3$ times. When tested for their ability to produce cells other than T lymphocytes, they were multipotent, like the hematopoietic stem cells. But, when compared with hematopoietic stem cells, the bone marrow Sca-2+ cells give rise to fewer colonies in the spleen. These functional tests also indicate that these precursors are at an intermediate stage between the bone marrow multipotent hematopoietic stem cells and the intrathymic low CD4 precursor cells. Our overall conclusion is that the bone marrow population most closely resembling the intrathymic low CD4 precursor is the Lin–Thy-1+HSA–Sca-2+ population. However, these cells appear to be multipotent rather than lymphoid committed. By way of qualification, it should be noted that we cannot exclude the possibility that a small number (≤1%) of Sca-2+ stem cell contaminants account for the thymus reconstituting activity of the Sca-2+ cells. However, this would require that the Sca-2+ cells giving rise to the spleen colonies (which are too many and too small to be derived from any Sca-2+ contaminants) had no or very low thymus reconstituting activity, which is an interesting alternative currently being investigated further. The presence of contaminants can only be resolved by further extensive limit dilution experiments.

In recent years, there have been a number of reports giving evidence for a common precursor for B and myeloid cells in the bone marrow. These reports implied that T lymphocytes develop along a separate pathway from B cells. Cumano et al isolated cells from murine fetal liver, and Ohara et al isolated cells from bone marrow, which developed in vitro into B and myeloid cells. Other reports have documented cell lines that can be directed to become either B cells or myeloid cells. However, in none of these reports was the T-cell potential of the cells in question adequately tested. Because T-lymphocyte development is a multistep process demanding a set of inducing factors, the T-cell potential of the cells under study may not have been expressed in the culture conditions used in these studies. It therefore remains possible that these B/myeloid precursors could also have developed into T lymphocytes, and so, in essence, were multipotent. Therefore, these reports would not exclude a further stage of development, like the intrathymic low CD4 precursors, from which only T and B lymphocytes would be produced. Still, it cannot be excluded is that a variety of partially committed precursors can develop, depending on the microenvironment in which they form. The resolution of these issues awaits further experiments.

### Table 3. Lymphoid Tissue Reconstitution 4 Weeks After IV Transfer of Bone Marrow or Thymic Precursors

<table>
<thead>
<tr>
<th>Cells Transferred</th>
<th>Tissue Analyzed</th>
<th>Donor-Derived Cells (Ly 5.2+) (%)</th>
<th>Percentage of Donor-Derived Cells Expressing Lineage Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sca-2+</td>
<td>Thymus</td>
<td>54</td>
<td>Thy-1+ : 98 &lt;0.2</td>
</tr>
<tr>
<td>Low CD4 precursor†</td>
<td>Thymus</td>
<td>1.4</td>
<td>B220+ : &lt;0.3</td>
</tr>
<tr>
<td>Sca-2-</td>
<td>Lymph nodes and spleen</td>
<td>38</td>
<td>Gr-1+ : &lt;0.3</td>
</tr>
<tr>
<td>Low CD4 precursors</td>
<td>Lymph nodes and spleen</td>
<td>9</td>
<td>Mac-1- : &lt;0.3</td>
</tr>
<tr>
<td>Sca-2+</td>
<td>Bone marrow</td>
<td>45</td>
<td>Thy-1+ : 0.9</td>
</tr>
<tr>
<td>Low CD4 precursors</td>
<td>Bone marrow</td>
<td>0.9</td>
<td>B220+ : 75 25 &lt;0.4</td>
</tr>
</tbody>
</table>

* Lin–Thy-1+HSA–Sca-2+ bone marrow precursors ($3.5 \times 10^5$ injected).† Intrathymic low CD4 precursors ($30 \times 10^5$ cells injected).

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REFERENCES


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