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Characterization of Thymic Progenitors in Adult Mouse Bone Marrow

S. Scott Perry, † L. Jeanne Pierce, ‡ William B. Slayton, § and Gerald J. Spangrude 2*†‡

Thymic cellularity is maintained throughout life by progenitor cells originating in the bone marrow. In this study, we describe adult mouse bone cells that exhibit several features characteristic of prothymocytes. These include 1) rapid thymic engraftment kinetics following i.v. transplantation, 2) dramatic expansion of thymic progeny, and 3) limited production of hemopoietic progeny other than thymocytes. The adult mouse bone marrow population that is depleted of cells expressing any of a panel of lineage-specific Ags, stem cell Ag-1 positive, and not expressing the Thy1.1 Ag (Thy1.1-) (Thy1.1- progenitors) can repopulate the thymus 9 days more rapidly than can hemopoietic stem cells, a rate of thymic repopulation approaching that observed with transplanted thymocytes. Additionally, Thy1.1+ progenitors expand prolifically to generate thymocyte progeny comparable in absolute numbers to those observed from parallel hemopoietic stem cell transplants, and provide a source of progenitors that spans multiple waves of thymic seeding. Nevertheless, the Thy1.1+ population yields relatively few B cells and rare myeloid progeny posttransplant. These observations describe the phenotype of an adult mouse bone marrow population highly enriched for rapidly engrafting, long-term thymocyte progenitors. Furthermore, they note disparity in B and T cell expansion from this lymphoid progenitor population and suggest that it contains the progenitor primarily responsible for seeding the thymus throughout life. The Journal of Immunology, 2003, 170: 1877–1886.

The nature of thymic progenitors in primary hemopoietic organs has been the subject of considerable investigation. Early forays into this arena compared the engraftment potentials of either whole bone marrow or thymocytes in irradiated mice. These experiments found that either intrathymic (i.t.) or i.v. transfer of whole thymocytes produced similar thymic engraftment kinetics, both resulting in detectable thymic progeny in about 10 days that persisted for an additional 10–15 days. Likewise, either i.t. or i.v. transfer of whole bone marrow produced comparable rates of thymic colonization; however, two distinct differences from thymocyte transplant were apparent. First, bone marrow transfer produced detectable thymic colonization 5 days later than did thymocyte transplant, and second, only bone marrow transferred i.v. produced sustained thymic colonization for the life of the host animal (1, 2).

These and similar observations have led to the conclusions that the thymus does not provide a suitable environment for renewal of thymic progenitors and that the thymus is seeded by bone marrow progenitors throughout life. Furthermore, it is evident from these observations that progenitors within the thymus do not produce a source of persistent engraftment, even when given access to the marrow by i.v. transplant. The bone marrow-derived thymic progenitor predicted by these conclusions has yet to be identified in adult mice; however, T lineage-restricted progenitors have been observed in fetal blood (3) and fetal liver (4). A T/NK bipotent precursor has also been recently identified in fetal liver (5). These fetal progenitors have been observed at equal frequencies in both normal and athymic mice, indicating that, during development, T lineage commitment can occur before thymic colonization. However, it is unclear whether such a T-committed progenitor exists in adult mouse bone marrow.

The adult mouse thymus harbors a population of progenitors that can produce T cells, B cells, NK cells, and thymic dendritic cells in culture (6–8). The multipotent potential of this population, identified as CD117+CD44+Lin− bone marrow cells, suggests that it may be a progenitor predicted by these conclusions, having the ability to produce both T cells and B cells. The multipotent progenitor seeds the thymus, where it further differentiates and commits to the T lineage (9, 10). However, clonal analysis has not been reported from this thymic fraction, leaving open the possibility that multiple lineage-committed progenitors may reside separately within this population. Even if this is not the case, the presence of a multipotent population in the adult thymus does not preclude the possibility that the thymus is also seeded by progenitors already committed to the T lineage such as those observed during fetal hemopoiesis.

We have used an in vivo transplant model to test the hypothesis that a T lineage-committed progenitor exists in adult mouse bone marrow. To identify progenitors with the rapid and sustained engraftment activity to be expected from a prothymocyte bone marrow population, we transplanted increasingly restrictive subsets of bone marrow i.v. into radiation-conditioned hosts and followed engraftment of differentiated hemopoietic lineages in both the marrow and thymus. For initial transplant studies, we subdivided the nucleated bone marrow population lacking expression of lineage-associated markers (Lin−) marrow) into either stem cell Ag-1

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(Sca-1)-expressing (Sca-1\textsuperscript{+}) or non-expressing (Sca-1\textsuperscript{-}) fractions, and subsequently tested progressively refined subsets of those fractions showing the greater and more rapid thymic engraftment potential. The resulting data show that cells with prothymocyte characteristics can be successfully separated from hemopoietic stem cells (HSC), and support the existence of a novel progenitor within the Sca-1\textsuperscript{c-Kihtight} bone marrow compartment possessing substantial prothymocyte properties. Accordingly, we conclude that at least some degree of commitment to the T cell lineage occurs before marrow egress.

**Materials and Methods**

**Mouse strains**

C57BL/6 (B6) and B6-Thy-1.1-Ly-5.1 congenic mice were bred and maintained at the Animal Resource Center facility of the University of Utah. Mice were used between 4 and 24 wk of age. All animals were maintained on autoclaved, acidified water (pH 2.5), and autoclaved chow.

**Antibodies**

mAbs against CD8 (53-6.7), CD11b (M1/70), erythrocytes (TER-119), Ly-6G (RB6-8C5), CD3 (KT3-1.1), CD5 (53-7.3), CD2 (Rm2.2), CD45R (RA3-6B2), Thy-1.1 (19XE5), CD19 (1D3), and Ly-5.1 (A20) were purried from media of cultured hybridoma cell lines. mAbs used for cell surface staining of Ly-5.1, Thy-1.1, and Ly-6G were conjugated with biotin, PE, or FITC in our laboratory. Biotinylated Abs were secondarily stained with either PE-streptavidin (SAv) (Biomedia, Foster City, CA) or FITC-SAv (Biomedia). In addition, PE-conjugated mAbs to Sca-1 and CD19, and allophycocyanin-conjugated c-Kit (allophycocyanin-c-Kit) Ab were purchased from BD Pharmingen (San Diego, CA).

**Preparation of bone marrow cells and isolation of hemopoietic stem and progenitor cell populations**

The procedure for the preparation of bone marrow cells for sorting has been previously described (11). Briefly, bone marrow cells were isolated from the femurs and tibia of 4- to 8-wk-old B6-Thy1.1-Ly-5.1 mice, and the erythrocytes were lysed in an ammonium chloride solution. The cells were incubated in a lineage mixture containing optimized concentrations of Abs to CD2, CD3, CD5, CD8, CD11b, Ly-6G, TERT119, CD45R, and CD19. Lineage depletion was conducted by two successive incubations of the bone marrow cells in sheep anti-rat Ig-coupled magnetic beads (Dynal, Oslo, Norway). The Lin\textsuperscript{−} cells were stained with PE-Sca-1 and sorted using a FACSVantage (BD Immunocytometry Systems, San Jose, CA) using enrichment mode. To ensure that both Sca-1\textsuperscript{high} and Sca-1\textsuperscript{low} bone marrow fractions were collected, the Sca-1\textsuperscript{+} gate was set to collect all events with PE emissions above background as determined by negative control stains for each sorting experiment. Dead cells were excluded from all analyses and sorts by gating on forward scatter and propidium iodide (PI; Molecular Probes, Eugene, OR) staining. The sorted Lin\textsuperscript{−} Sca-1\textsuperscript{+} cells were pelleted, stained with allophycocyanin-c-Kit and FITC-Thy-1.1, and resorted using normal mode into Thy1.1\textsuperscript{high} and Thy1.1\textsuperscript{low} populations. Further Thy1.1\textsuperscript{−} subsets were also sorted based on c-Kit expression for specific experiments as noted in Results. For experiments where Sca-1\textsuperscript{+} cells were assayed against Sca-1\textsuperscript{−} cells, the PE-Sca-1\textsuperscript{−} stained Lin\textsuperscript{−} marrow was sorted using the FACSVantage set at normal mode, and the Sca-1\textsuperscript{−} and Sca-1\textsuperscript{+} fractions were collected. For all experiments, sorting steps were done using the FACSVantage, and cells were sorted directly onto 20 μl of 100% FCS (HyClone Laboratories, Logan, UT) contained in the wells of a round-bottom 96-well microtiter plate. An aliquot of each sorted cell population was taken for reanalysis to verify its purity. The cell concentrations of the final aliquots were verified by hemocytometer, and cell viability was assessed by culturing an aliquot in methycellulose, as described below.

**Methylcellulose assays**

To verify the cell viability and concentration in the solutions of sorted cells transplanted into host mice, aliquots were cultured in methycellulose at a plating density of ~100 cells/35-mm culture dish. Each milliliter of culture medium contained alpha-MEM (Life Technologies, Gaithersburg, MD), 1.2% methylcellulose (Shinetsu, Tokyo, Japan), 30% FCS, 1% deionized BSA (Sigma-Aldrich, St. Louis, MO), 0.1 mM 2-ME (Malinkrodt Chemical, Chesterfield, MO) supplemented with optimized concentrations of the cytokines listed below. Culture dishes were incubated at 37°C and infused with 5% CO\textsubscript{2}. The number of colonies was counted using an inverted microscope after 7 days of culture, and the cloning efficiencies were compared with established standards for each population. Four to six plates were scored for each population transplanted.

CFU-culture assays testing the lineage potential of Lin\textsuperscript{−} Sca-1\textsuperscript{+} Thy1.1\textsuperscript{−} c-Kihtight cells followed the same procedure and cytokine stimulation as described above, except only 50 cells were seeded in each culture dish, allowing individual colonies to be separately collected and assayed by immunofluorescent staining and flow cytometry. Twenty-four colonies were enumerated at each time point specified in Results through suctioning the colony with surrounding methycellulose into a 200-μl pipette. This was then transferred to an appropriate tube and washed once in HBSS supplemented with 5% FCS (HSF). Cells were then stained with PI and PE-labeled anti-CD19 and FITC-labeled anti-Ly-6G Abs as described below for the in vivo assessment of progenitor potential.

**Cytokines**

Steel factor (SLT) and G-CSF were a kind gift from Gemini Science (San Diego, CA), a subsidiary of Kirin Pharmaceuticals (Tokyo, Japan). Flt3 ligand and IL-6 were kindly provided by Immunex (Seattle, WA). Recombinant human erythropoietin was purchased from Ortho-McNeil Pharmaceuticals (Raritan, NJ). Recombinant murine IL-3 and IL-7 were purchased from PeproTech (Rocky Hill, NJ). The cytokines were used at the following concentrations in all methylcellulose assays: STL 100 ng/ml; G-CSF 10 ng/ml; Flt3 ligand, 75 ng/ml; IL-6, 20 ng/ml; erythropoietin, 5 U/ml; IL-3, 10 ng/ml; and IL-7, 10 ng/ml.

**In vivo assessment of progenitor potential**

All recipient mice were exposed to a single 6.5-Gy dose of radiation from a 137Cs source (Mark I gamma irradiator; J. L. Shepherd, Glendale, CA). Several hours later, mice were anesthetized with isoflurane (Isove, Vedco, St. Joseph, MO), and the purified cell population was infused i.v. via the retroorbital sinus. Host animals were 6- to 24-wk-old B6 mice, while donor cells were obtained from 4- to 8-wk-old B6-Thy-1.1-Ly-5.1 mice. Recipient animals were maintained on oral neomycin sulfate (Biosol, 2 mg/ml; Upjohn, Kalamazoo, MI) for 2 wk after irradiation and transplant. At the time points indicated, the recipient B6 mice were sacrificed, and the thymic lobe and the marrow from one femur were collected separately. Erythrocytes in the marrow samples were lysed by incubation in ammonium chloride potassium solution, and the total nucleated cells in each sample were assessed by analyzing an aliquot with a Sereno System 9100 automated hematology analyzer (ABX Diagnostics, Irvine, CA).

Bone marrow samples were stained in a two-step process. Cells were first suspended at $5 \times 10^7$ cells/ml in HSF and reacted with a saturating solution of biotin-conjugated anti-Ly-5.1 Abs to label donor-derived cells. After incubating 20 min on ice, the cells were washed in HSF and resuspended in a saturating solution of FITC-SAv and PE-labeled anti-CD19 or anti-Ly-6G Ab. After another 20-min incubation, cells were washed and resuspended at $10^7$ cells/ml in an optimized concentration of PI in HSF. Donor-derived thymocytes were similarly identified in the thymic samples by a first-step reaction with biotin anti-Ly-5.1 and a secondary reaction with both PE-SAv and FITC-labeled anti-Thy1.1. All samples were analyzed by flow cytometry on a FACSscan analyzer (BD Immunocytometry Systems, San Jose, CA). Cell viability was assessed by gating on PI-negative events, and the absolute numbers of donor-derived B cells, thymocytes, or granulocytes were determined by multiplying the percentage of viable donor-derived cells in each lineage as observed with flow cytometry by the total number of cells in that tissue sample.

**Results**

Rapidly engrafting thymic progenitors in mouse bone marrow are confined to the Sca-1\textsuperscript{+} compartment

To test the hypothesis that a T lineage-committed progenitor resides in adult mouse bone marrow, we used i.v. transplantation into sublethally irradiated mice to systematically analyze the engraftment potential of increasingly limited bone marrow cell subsets. The initial steps in this process focused on the subsets of Lin\textsuperscript{−} marrow separated by Sca-1 expression. Sca-1 is a GPI-linked extracellular membrane protein expressed on primitive hemopoietic progenitors in mouse bone marrow (12, 13). Lin\textsuperscript{−} Sca-1\textsuperscript{+} cells are ~13-fold more frequent in the marrow of B6 mice than are their Sca-1\textsuperscript{+} counterparts. To test for the
presence of thymic progenitors within these compartments, we transplanted either 900 Lin- Sca-1+ cells or a frequency equivalent of 12,000 Lin- Sca-1+ cells from B6-Ly-5.1-Thy1.1 mice i.v. into sublethally irradiated (6.5 Gy) B6 mice. At 20 days posttransplant, both thymic lobes were collected from each animal and examined by immunofluorescent staining and multiparameter flow cytometry for donor-derived reconstitution. One femur from each animal was also collected in this and subsequent experiments and assayed for B cell and myeloid reconstitution to assess the degree of lineage commitment within the targeted marrow population. Thymocyte engraftment was determined by assaying thymic lobes separately for cells coexpressing the donor markers Ly-5.1 and Thy1.1. Likewise, B cell reconstitution was assessed by examining thymic lobes separately for cells coexpressing Thy1.1 and H-2d. Likewise, B cell reconstitution was assessed by examining thymic lobes separately for cells coexpressing Thy1.1 and H-2d.

Animals transplanted with Sca-1+ cells did not show reconstitution that exceeded 105 cells/organ of any of these three lineages. In contrast, reconstitution was common among animals receiving Sca-1- cells (Table I). Repeating this experiment at 2-fold higher doses resulted in 100% engraftment of all three lineages in mice receiving Sca-1+ cells, but still did not produce detectable engraftment among Sca-1- cell recipients. Consequently, we concluded that all the highly proliferative thymic progenitors within Lin- marrow bone marrow reside in the Sca-1+ fraction, and focused further investigations on subsets of this compartment.

### Highly proliferative thymic progenitors can be separated from HSC based on Thy1.1 expression

The Thy1.1 allele of CD90 has been shown to define functionally distinct subsets within the Lin- Sca-1+ bone marrow compartment. Cells of the Lin- Sca-1+ fraction express Thy1.1 either moderately (Thy1.1low) or not at all (Thy1.1+; Fig. 1A). The Thy1.1low population has been shown to be highly enriched for HSC (12, 14). We have previously observed rapid and substantial B cell engraftment in peripheral blood and bone marrow posttransplant with the Thy1.1+ fraction of this compartment; however, erythrocyte and platelet reconstitution were absent, and Thy1.1+ progenitors did not rescue lethally irradiated mice (15). To evaluate the prothymocyte potential of these Sca-1- fractions, we transplanted either 107 Thy1.1+ progenitors or 103 Thy1.1low HSC into parallel cohorts of mice and assayed them as described above. Animals transplanted with Thy1.1+ progenitors showed a considerable degree of thymic colonization at day 21 that was dramatically absent in animals transplanted with Thy1.1low HSC (Fig. 1B). In some cases, the expansion produced by Thy1.1+ progenitors reached >107 cells/thymic lobe, while the donor-derived thymocyte level in animals receiving HSC did not exceed 105 cells/lobe. The data from Fig. 1B indicate that a highly proliferative thymic progenitor can be separated from the Thy1.1low HSC compartment, and narrow the Lin- compartment that might contain a bone marrow prothymocyte to those progenitors that are both Sca-1- and Thy1.1-.

### The Thy1.1low population includes progenitors capable of thymic reenrollment with kinetics comparable to a thymocyte graft

Several laboratories have demonstrated that thymic engraftment can be observed at time points earlier than day 21 if large numbers of unseparated bone marrow cells are transplanted i.v. (2, 16, 17). To confirm that the Thy1.1- compartment contains the progenitors responsible for early thymic reenrollment, we performed a kinetic analysis. Fig. 1C presents pooled data from eight such experiments, representing 82 transplant recipients. These experiments showed that i.v. transplantation of Thy1.1- cells mediated thymocyte engraftment by day 12, 9 days earlier than when engraftment was first observed from HSC on day 21. An expansion level of 107

### Table I. Engraftment posttransplant with selected progenitor subsets

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<th>Population</th>
<th>Cell Dose</th>
<th>Day Posttransplant Analyzed</th>
<th>Number of Mice Analyzed</th>
<th>Percentage of positive1a thymic lobes</th>
<th>Mean2 expansion size (×105)</th>
<th>Percentage of positive femurs</th>
<th>Mean expansion size (×105)</th>
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<td>29.8 ± 4.3</td>
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1a Sublethally irradiated mice were transplanted i.v. with the indicated progenitors from congenic donors. At the indicated times, both thymic lobes were collected separately from each animal and assayed by immunofluorescent staining and flow cytometry for cells coexpressing the congenic donor marker and the indicated lineage marker.

1b Organs were defined as positive when containing ≥105 donor-derived cells of the indicated lineage.

1c Average number of donor-derived cells of the indicated lineage per organ for positive organs.

1d Error values indicate SEM. Where no error is given, only one organ from the group of animals assayed at that time point was found to be positive.
donor-derived thymocytes-engrafted thymic lobe was characterized by day 19 in animals transplanted with Thy1.1\(^{low}\) progenitors, which was also 9 days sooner than achieved with HSC transplants. By day 21, mice transplanted with Thy1.1\(^{low}\) progenitors continued to maintain significantly more donor-derived thymocytes per engrafted lobe than did HSC-transplanted mice (p = 0.01).

To compare the engraftment kinetics of Thy1.1\(^{low}\) progenitors with a more committed T lineage progenitor population, we evaluated thymic engraftment following transplants of normal, unseparated thymocytes. Twenty-five radiation-conditioned hosts were transplanted i.v. with a saturating dose (2.5 \(\times\) 10\(^7\) cells) of thymocytes and evaluated for thymic engraftment over time. As shown in Fig. 1C, thymic engraftment after thymocyte (○) transplant preceded engraftment of Thy1.1\(^{low}\) progenitors (□) by 4 days. The wave of engraftment generated by transplanted thymocytes peaked at a 10-fold lower number of cells than was observed from either Thy1.1\(^{low}\) progenitor or HSC transplants. Further, engraftment persisted for only 11 days, extinguishing before engraftment from HSC transplant was evident. The limited persistence of thymic engraftment observed following thymocyte transplant is in contrast to the maintenance of engraftment following transplantation of Thy1.1\(^{low}\) progenitors. To evaluate the persistence of thymocytes derived from Thy1.1\(^{low}\) progenitors, we extended our assay to include time points as late as days 42 and 56. At both of these assay points, we continued to observe populations of thymocytes derived from the Thy1.1\(^{low}\) progenitors, although the absolute number of donor-derived cells diminished after day 21 (Table I). Nonetheless, of the 10 thymic lobes assayed on day 56, 3 contained >10\(^5\) donor-derived thymocytes.

i.t. transfer of normal, unseparated bone marrow cells into radiation-conditioned mice results in thymic colonization that persists for <25 days posttransplant (2). Accordingly, the data presented in Table I indicate that a subset of thymic progenitors within the Thy1.1\(^{low}\) population first engrafts in the marrow, and then proceeds to seed the thymus for at least twice the duration of an i.t. transplant. The characteristics of thymic engraftment by Thy1.1\(^{low}\) progenitors compared with HSC and to normal thymocytes confirm that adult mouse bone marrow contains a prothymocyte progenitor capable of thymic engraftment with kinetics comparable to thymocytes and with proliferative potential comparable to HSC.

The extent of hemopoietic engraftment by the Thy1.1\(^{low}\) population is distinct from that of HSC

Previous studies of the progenitor potential within the Thy1.1\(^{low}\) bone marrow population suggested that the prothymocyte activity would be accompanied by other lineage potentials (15, 18, 19). To investigate this possibility, we evaluated a marrow sample from each transplant recipient for engraftment of B lymphocyte and myeloid lineages (Table I). This analysis revealed that Thy1.1\(^{low}\) progenitors mediate a robust, early wave of B cell engraftment that began at ~day 9 and peaked by day 12 before tapering off to lower levels of consistent engraftment that persisted through at least day 56. This pattern was distinctly different from the reconstitution of Ly-6G\(^+\) lineages, which showed no early engraftment and only sporadic engraftment after day 19 (Table I).

The pattern of marrow engraftment following i.v. transplant of Thy1.1\(^{low}\) progenitors contrasted sharply with that observed in parallel transplants of Thy1.1\(^{low}\) HSC, highlighting the difference in hemopoietic potential between these two populations. Specifically, Thy1.1\(^{low}\) HSC transplants did not produce >10\(^5\) B cells/femur until day 17, 8 days later than achieved by Thy1.1\(^{low}\) transplant. Conversely, Thy1.1\(^{low}\) HSC on average produced >2 \(\times\) 10\(^5\) Ly-6G\(^+\) cells/femur at each time point assayed, beginning at day 7, while only 2 of 88 mice transplanted with Thy1.1\(^{low}\) progenitors achieved this level of engraftment, and not until days 21 and 28. Furthermore, transplant of 10\(^3\) Thy1.1\(^{low}\) HSC produced engraftment of at least one lineage in all mice evaluated, regardless of the time posttransplant. In contrast, 20% of mice transplanted with the same number of Thy1.1\(^{low}\) progenitors showed no detectable engraftment. Fig. 2A resolves this statistic as a function of time. Peak engraftment frequency (91.7%) was not achieved among Thy1.1\(^{low}\) progenitor recipients until day 21, and then declined persistently.The Thy1.1\(^{low}\) population and its engraftment kinetics relative to Thy1.1\(^{low}\) HSC. A, Representative profile of Thy1.1 and c-Kit expression within the Lin\(^{−}\)Sca-1\(^{−}\) compartment of 5-wk-old B6-Thy1.1-Ly-5.1 mouse bone marrow. Guides indicate the populations tested in these studies. Inset indicates the percentages of Lin\(^{−}\)Sca-1\(^{−}\) cells that fall into each population. Percentages were averaged from nine replicate sorting experiments representing marrow pooled from 74 total donor mice. B, Thy1.1\(^{low}\) progenitors and Thy1.1\(^{low}\) HSC were collected from B6-Thy1.1-Ly-5.1 donor mice and transferred i.v. into sublethally irradiated (6.5 Gy) B6 host mice. Each host animal received either 10\(^5\) Thy1.1\(^{low}\) progenitors or 10\(^5\) Thy1.1\(^{low}\) HSC. Thymic lobes were collected individually from host mice at 21 days posttransplant and assayed by immunofluorescent staining and flow cytometry for donor-derived (Ly-5.1\(^{+}\)) thymocytes. Shown are the numbers of donor-derived thymocytes observed per thymic lobe in mice transplanted with either HSC (■) or Thy1.1\(^{low}\) progenitors (□). Lobes with <10\(^4\) donor-derived thymocytes were considered negative in this assay. C, Hosts were conditioned as described for B and transplanted with either 10\(^5\) Thy1.1\(^{low}\) progenitors, 10\(^5\) Thy1.1\(^{low}\) HSC, or 2.5 \(\times\) 10\(^5\) whole thymocytes. Thymic lobes were collected from host animals at the times indicated and assayed as described for B. Animals received Thy1.1\(^{low}\) progenitors (□), whole thymocytes (○), or HSC (●). Each symbol represents the mean donor-derived thymocytes per positive thymic lobe observed at that time point. Error bars represent SEM. Three to 15 mice were assayed for each data point shown.
for the duration of the assay. Meanwhile, Thy1.1low HSC mediated increasing engraftment frequencies and lineage expansion throughout the assay (Table I).

The Thy1.1 population contains multiple progenitors with distinct lineage potentials

The combined examination of recipient thymus and marrow for thymocyte, B cell, and myeloid engraftment over time revealed a specific profile of lineages developing from Thy1.1 progenitors following i.v. transplantation. A common profile seen in hosts transplanted with Thy1.1 progenitors was an early burst of B cell engraftment in the marrow that was not accompanied by either thymocyte or myeloid engraftment (Fig. 2B). This burst began at day 9 and lasted for 10 days before dropping off rapidly at day 19. Mice with thymocytes as the only progeny of the transplanted cells were observed from day 19 through day 42 (Fig. 2B). Underlying these two curves was a biphasic wave of engraftment where both B cells and thymocytes were present, but myeloid cells were not detectable. This wave was first observed on day 12 and persisted through day 56 (Fig. 2C).

The coincidence of the thymocyte-only engraftment wave (Fig. 2B) with the second peak in the B cell/thymocyte mixed engraftment pattern (Fig. 2C) suggests that these observations may be the result of a single, bipotent progenitor. The thymocyte-only engraftment may be the result of this progenitor homing directly to the thymus posttransplant, where its potential would be restricted to thymocyte development. Likewise, the B/thymocyte mixed engraftment may represent this progenitor engrafting in the marrow, where physiological cues would allow daughter cells to either exit the marrow, home to the thymus and develop as thymocytes, or remain in the marrow and follow B lineage development. The initial peak of combined B cell/thymocyte engraftment appears nearly 10 days sooner than is characteristically observed for thymocytes following i.v. transfer of whole bone marrow (2), and appears essentially as rapidly as does thymocyte engraftment following i.v. transfer of whole thymocytes (compare Fig. 2C to 1C). This may represent the activity of a rare thymocyte-committed progenitor homing directly to the thymus, with readout in these assays overlapping with a more common B lineage-committed progenitor. Further study using clonal analysis will be required to test this hypothesis.

Fig. 2C also shows that a small number of mice exhibited B cell and myeloid reconstitution in the absence of thymocyte reconstitution between days 19 and 24. At the same time, a few mice exhibited simultaneous engraftment of thymocyte, B cell, and myeloid lineages. Both the tripotent and the lymphoid-restricted engraftment patterns were observed at 56 days, the endpoint of the analysis. If these activities represent separate progenitor populations, they may both be capable of the long-term expansion observed with Thy1.1 progenitors in these assays. Kawamoto et al. (4) and others (20–22) have used clonal assays to demonstrate a bipotent B/myeloid progenitor in murine fetal liver and in adult bone marrow (23). Our observations of reconstitution restricted to B cell and myeloid lineages are consistent with these studies; however, definitive demonstration of the in vivo activity of the bipotent B/myeloid progenitor will require prospective isolation of these cells separate from other progenitors within the Thy1.1 progenitor population.

Levels of c-Kit expression define lymphoid-restricted progenitors within the Thy1.1 compartment

Cells within the Thy1.1 population express the STL cytokine receptor c-Kit at varying intensities (Fig. 1A). Several studies have identified lymphoid-restricted progenitors within the Lin– mouse bone marrow fraction low in c-Kit expression (18, 19, 24), implying that levels of c-Kit expression may indicate developmental and functional distinctions among progenitor populations. Accordingly, we reasoned that this marker might prove useful in resolving the progenitor activities we had observed following transplantation of the unseparated Thy1.1 population. To test this hypothesis, we separated the Thy1.1 population into three subpopulations based on c-Kit expression (Fig. 1A). Over three experiments, 500-1500 cells from these fractions were transplanted i.v. into sublethally irradiated mice. Animals were subsequently assayed for engraftment as described above, at time points both before and after day 21, when significant changes in progenitor activity had been previously observed (Fig. 2). Results from these assays showed that Thy1.1 progenitors lacking c-Kit expression produced a limited number of B cells in the marrow but lacked detectable thymocyte engraftment ability (Fig. 3 and data not shown). Both c-Kit-bright and -low populations showed comparable thymocyte and marrow B cell engraftment frequencies at short-term time points before
myeloid engraftment developed (Fig. 3A). However, c-Kitbright cells showed the vast majority of thymocyte engraftment potential and all of the myeloid and B lineage potential detectable in the marrow at later time points (Fig. 3B).

The c-Kitbright fraction of the Thy1.1+ population is enriched for specific progenitors

The high frequency of both long- and short-term thymocyte progenitors within the c-Kitbright fraction of the Thy1.1+ population prompted further analysis of this subset. Over four experiments, 45 mice were sublethally irradiated and each transplanted i.v. with 10^3 Thy1.1+ c-Kitbright progenitors, and then followed for B cell, myeloid, and thymocyte engraftment as described before. Between days 11 and 56 posttransplant, these grafts had expanded to >10^5 cells in at least one of the three lineages assayed in all but three mice, indicating that the c-Kitbright fraction was considerably enriched over the unseparated Thy1.1+ compartment for engraftable progenitors. The c-Kitbright fraction also produced consecutive waves of first B cell and then thymocyte-restricted engraftment at frequencies relatively proportional to those observed from transplant of the unseparated Thy1.1+ population (Fig. 4A, compare to 2B). We have previously characterized a subset of AA4.1+ committed B cell progenitors within the Thy1.1+ progenitor pool (19). However, the B lineage engraftment observed in these transplant experiments is unlikely to be due to the AA4.1+ cells, because this subset is restricted to the c-Kitlow fraction of the Thy1.1+ progenitor pool and would have been depleted from the c-Kitbright population transplanted in these studies. The observation of engraftment restricted to the B lineage indicates that a separate progenitor with rapid B cell expansion potential resides within the c-Kitbright fraction.

The combination of hemopoietic lineages observed after i.v. transplantation of Thy1.1+ c-Kitbright progenitors contrasted remarkably with those seen after transplant of the greater Thy1.1+ population. Most notably, the frequency of coincident thymocyte, granulocyte, and marrow B cell engraftment increased nearly 8-fold, from 3 of 88 mice (3.4%) following transplant of Thy1.1+ progenitors to 11 of 45 mice (24%) following transplant of the Thy1.1+ c-Kitbright subset. Conversely, the incidence of combined thymocyte and marrow B cell engraftment in the absence of detectable myeloid contribution decreased from 25% (22 of 88 animals) with unseparated progenitors to 16% of mice (7 of 45) receiving the c-Kitbright fraction (Figs. 2C and 4B). This correlates with depletion of progenitors positive for expression of the IL-7Rα-chain (IL-7Rα), which are mostly c-Kitlow and constitute <10% of the Thy1.1+ c-Kitbright population (Fig. 4C). Kondo et al. (18) demonstrated that the Thy1.1+ IL-7Rα+ fraction contains a common lymphoid progenitor (CLP) capable of clonally generating both T and B cells at high frequencies. Engraftment of both B cells and granulocytes in marrow in the absence of thymic T cells was observed only at a single time point (15 days posttransplant).

As a control for each of the transplant experiments, aliquots of freshly sorted cells were cultured in methylcellulose with a complex mixture of cytokines to verify the viability of the transplanted...
population. The c-Kit$^{bright}$ fraction proved to be highly clonogenic, with cloning efficiencies increasing with time from 24.5 ± 6.5% on day 5 to a peak of 40.4 ± 6.8% on day 12. Interestingly, the morphology of the colonies generated in these cultures suggested that the majority were myeloid in nature. Because this contrasted remarkably with the hemopoietic lineages generated posttransplant with the same cells, we analyzed a representative fraction of colonies by immunofluorescent staining and flow cytometry. Over three separate experiments, individual colonies were randomly selected between days 5 and 12 of culture and analyzed for viability by PI exclusion as well as for Ly-6G and CD19 expression. Of the 144 colonies assayed, 101 contained >30 viable cells. Ninety-one of these were composed of Ly-6G$^+$ myeloid cells, while seven consisted of CD19$^+$ B cells, and two colonies contained both lineages (data not shown). In contrast, we have previously shown that Thy.1$^+$ c-Kit$^{low}$ cells are largely depleted of myeloid colony-forming potential (19). Collectively, the kinetic analysis of lineage engraftment after transplantation of Thy.1$^+$ c-Kit$^{bright}$ progenitors indicates that this population contains the major B cell and myeloid potentials within the Thy.1$^+$ population.

The Thy.1$^+$ c-Kit$^{bright}$ population is enriched for rapidly engrafting, highly proliferative thymocyte progenitors

Analysis of thymocyte engraftment kinetics after i.v. transplantation of Thy.1$^+$ c-Kit$^{bright}$ progenitors showed that this population generated $10^7$ thymocytes/thymic lobe within 14 days posttransplant. These kinetics are similar to those of the unseparated Thy.1$^+$ population, and only 3 days slower than the time required by thymocytes to achieve this degree of engraftment (Fig. 4D). Progeny of transplanted Thy.1$^+$ c-Kit$^{bright}$ cells exceeded $10^7$ thymocytes/thymic lobe by 3 wk posttransplant, essentially repopulating the entire lobe despite competition from endogenous progenitors in the sublethally irradiated mice.

To further characterize the lineage potential of Thy.1$^+$ c-Kit$^{bright}$ progenitor cells, we transplanted 31 additional mice over four experiments with incremental doses of ranging from 50 to 1100 cells. Mice were assayed for marrow and thymic engraftment between 4 and 5 wk posttransplant. Among recipients of these transplants, the most common outcome was development of thymocytes in the absence of marrow engraftment (22.6%; Table II), particularly among animals receiving transplants of the lowest cell doses (Table III). Of 5 mice transplanted with 100 Thy.1$^+$ c-Kit$^{bright}$ progenitors, 3 showed engraftment and 2 had donor-derived thymocytes without detectable marrow proliferation in either B or myeloid lineages. Two of 2 mice transplanted with 200 cells each showed thymic engraftment in the absence of marrow engraftment (Table III). Animals receiving grafts of only 50 progenitors showed no detectable progeny. The remarkable rate of thymocyte engraftment from relatively few transplanted cells demonstrates that the Thy.1$^+$ c-Kit$^{bright}$ bone marrow compartment is considerably enriched for progenitors with prothymocyte characteristics. The only other engraftment noted at doses of 50–200 cells was a single mouse with donor-derived thymocytes and myeloid cells. Overall, 10 of 31 mice showed no detectable engraftment, and all of these were confined to cohorts receiving 500 or fewer cells. Doses of 675 cells or more were successful in producing engraftment of at least one lineage in all mice.

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<th>Table II. Thymic and marrow engraftment resulting from transplant with limiting doses of Thy.1$^+$ c-Kit$^{bright}$ progenitors$^a$</th>
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$^a$ Sublethally irradiated mice were transplanted i.v. with increasingly limiting doses of c-Kit$^{bright}$ Thy.1$^+$ progenitor cells and assayed for engraftment between days 28 and 37 posttransplant as described for Table I. Doses transplanted were 50, 100, 200, 350, 500, 675, and 1100 cells/mouse.

The only other engraftment noted at doses of 50–200 cells was a single mouse with donor-derived thymocytes and myeloid cells. Overall, 10 of 31 mice showed no detectable engraftment, and all of these were confined to cohorts receiving 500 or fewer cells. Doses of 675 cells or more were successful in producing engraftment of at least one lineage in all mice.

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<th>Table III. T-lineage restriction of engraftment following transplant of Thy.1$^+$ c-Kit$^{bright}$ progenitors$^a$</th>
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<td><strong>Cell Dose</strong></td>
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$^a$ Thymocyte engraftment among the 12 mice from Table II receiving the lowest doses of progenitors.

$^b$ Number of mice in each group.

$^c$ Percentage of transplanted mice having thymocyte engraftment alone or in combination with other lineages.

$^d$ Fraction of mice with T lineage engraftment in the absence of detectable marrow engraftment. One mouse at the 100-cell dose showed both thymocyte and myeloid engraftment.

We consistently observed sequential engraftment of marrow B lymphocytes followed by thymic T lymphocytes over time after i.v. transplantation of either total Thy.1$^+$ progenitors or the c-Kit$^{bright}$ subset (Figs. 2B and 4A). Interestingly, the second most common profile observed after i.v. transplantation of minimal numbers of progenitors from the Thy.1$^+$ c-Kit$^{bright}$ population was combined B cell and thymocyte engraftment in the absence of myeloid progeny. This was first observed at a dose of 350 Thy.1$^+$ c-Kit$^{bright}$ progenitors and occurred in roughly 19% of all mice transplanted (Table II). The relatively high frequency of this outcome despite the absence of nearly all IL-7$^+$ progenitors was somewhat surprising in light of the previously described CLP, and suggested that the c-Kit$^{bright}$ progenitor population contained a high frequency of B cell progenitors coincidentally engrafting with a separate population of thymocyte progenitors. Alternatively, the data could be interpreted to indicate that a bipotent B/T progenitor is present at high frequencies outside of the c-Kit$^{low}$IL-7$^+$ CLP compartment.

To address these possibilities, we analyzed peripheral lymph node lymphocytes in parallel with marrow and thymic samples obtained 4 wk posttransplant from sublethally irradiated mice transplanted i.v. with either 500 or 200 Thy.1$^+$ c-Kit$^{bright}$ progenitors as described above. According to previous results, thymocyte engraftment at this time was predicted to be prevalent while B cell engraftment was nearing extinction (Fig. 4A). We found that all but two mice had developed $>10^5$ donor-derived thymocytes/thymic lobe. However, only one animal showed $>10^5$ Thy.1$^+$ donor-derived T cells/peripheral lymph node. Conversely, no animal...
demonstrated detectable marrow engraftment of CD19<sup>+</sup> B cells, yet all animals showed robust peripheral lymph node colonization by donor-derived B cells, with all but one exceeding 10<sup>6</sup> donor CD19<sup>+</sup> cells/node (Table IV).

These results correlate with our previous finding that Thy1.1<sup>−</sup> progenitors produce few peripheral T cells (15) in contrast to their robust ability to reconstitute the thymus as shown in the present studies. This discrepancy is likely due to the high frequency of failure by nascent thymocytes to produce functional TCRs and the common elimination of nonproductive thymic clones by apoptosis in the thymus. More remarkably, these experiments show that most or all of the mice observed in previous experiments to show T lineage engraftment in apparent absence of B lymphocytes based on marrow analysis most likely had experienced a wave of B cell expansion earlier in the progression of the graft. This observation supports the hypothesis that the consecutive waves of B and T engraftment are produced from bipotent or multipotent Thy1.1<sup>−</sup> c-Kit<sup>bright</sup>IL-7R<sup>−</sup> progenitors rather than by separate lineage-restricted progenitors. This interpretation is strengthened by the relatively low cell dose (200–500 cells) at which the transplants shown in Table IV were performed.

The data from experiments using incremental doses of Thy1.1<sup>−</sup> c-Kit<sup>bright</sup> progenitors were analyzed to assess progenitor frequencies according to limiting dilution statistics (25); however, accurate estimates of either prothymocyte or B cell progenitor frequencies were not possible due to nonlinear results. This suggests that either multiple cell interactions are necessary to produce successful engraftment or more than one progenitor class present in the subset of cells can contribute to the engraftment readouts followed in these assays.

**Discussion**

The observations described in this study show that cells with prothymocyte characteristics can be successfully separated from and studied independently of HSC in adult murine bone marrow. The data presented show that the Lin<sup>−</sup>Sca-1<sup>−</sup>Thy1.1<sup>−</sup> bone marrow population includes progenitors that meet the definitive characteristics of prothymocytes, including 1) rapid thymic engraftment after i.v. transplantation, 2) dramatic expansion of thymic progeny, and 3) restricted production of hematopoietic lineages other than thymocytes. Additionally, progenitors within this population are capable of reconstituting the thymus with kinetics comparable to those of normal thymocytes as well as outcompeting reconstitution from endogenous host progenitors recovering from mild radiation conditioning. Thymic engraftment from the Thy1.1<sup>−</sup> population also persists to provide a source of progenitors that spans multiple waves of thymic seeding. Furthermore, through a process of elimination, these experiments show that the residence of prothymocytes within Lin<sup>−</sup> adult mouse bone marrow is limited to the Sca-1<sup>−</sup>Thy1.1<sup>−</sup> population.

Although the properties of this bone marrow compartment are remarkable and reminiscent of stem cell potential, they are distinctly different from HSC. The most striking of these differences is the accelerated rate of thymic engraftment evident after i.v. transplantation of Thy1.1<sup>−</sup> c-Kit<sup>bright</sup> progenitors (Figs. 1C and 4D). Other evidence includes the dramatic differences in lineages reconstituted between parallel transplants of Thy1.1<sup>−</sup> progenitors and HSC (Table I), as well as waning engraftment with time following transplant with both the unseparated Thy1.1<sup>−</sup> population and the c-Kit<sup>bright</sup> fraction (Figs. 2A, and 4, A and B) which was not observed following transplantation of HSC (Table I). This was most evident in multilineage engraftment from Thy1.1<sup>−</sup> c-Kit<sup>bright</sup> progenitors, where peak engraftment frequencies were rapidly eroded (Fig. 4B). Previous work has also shown that Thy1.1<sup>−</sup> progenitors do not rescue lethally irradiated mice (15).

Another population described as capable of rapid thymic reconstitution has been characterized as Lin<sup>−</sup>c-Kit<sup>−</sup> and high for retention of the mitochondrial dye rhodamine 123 (Rho<sup>high</sup>) (16). Thy1.1<sup>−</sup> progenitors also include Rho<sup>high</sup> cells (G. J. Spangrude, unpublished observation) and may therefore contain some of the cells reported as rapid thymic precursors; however, the rapidly engrafting Rho<sup>high</sup> population was reported to require 3 wk to colonize the thymus (6). Therefore, while this population may be included in the Thy1.1<sup>−</sup> fraction, the results reported in this study indicate that additional progenitors with more rapid thymic engraftment kinetics are also present (Fig. 1).

It must also be noted that the prothymocyte characteristics listed above were not successfully separated from other lineage potentials during this in vivo analysis. Thymic reconstitution was the most frequent result following Thy1.1<sup>−</sup> c-Kit<sup>bright</sup> transplant, with as few as 100 progenitors producing reproducible engraftment (Tables II and III); however, analysis of peripheral lymphoid tissues showed that apparent thymocyte-restricted engraftment was accompanied by at least a fleeting burst of earlier B cell expansion (Table IV). This was evident at transplant doses as low as 200 cells, and may to some degree explain why very early waves of B cell-restricted engraftment were consistently followed (with minimal overlap) by waves of apparent thymocyte-restricted engraftment (Figs. 2B and 4A). This observation also supports the hypothesis that these coordinated waves of apparent single-lineage engraftment are
mediated by the same progenitor, possibly responding to variations in hemopoietic stimuli during recovery from conditioning radiation. This hypothesis predicts a progenitor predisposed to the T cell lineage, but with sufficient developmental flexibility to adapt alternate lineage fates in response to the unusual circumstances of crisis hemopoiesis (10). Such a progenitor would also account for the nonlinear limiting dilution analysis results that precluded a precise estimate of the frequency for either T or B lineage progenitors within the Thy1.1 c-Kitbright compartment. Alternatively, the possibility that the transplanted population also contains a high frequency progenitor with rapid but limited B cell expansion potential could also explain both the kinetic observations and the nonlinear limiting dilution results. Further fractionation of the population coupled with clonal assays will be required to address these questions.

Combined B/thymocyte engraftment was among the most common engraftment patterns observed following transplant of unseparated Thy1.1 progenitors (Fig. 2C). This frequency was noticeably diminished following transplant of the c-Kitbright subset (Fig. 4B), an observation that correlates with the depletion of IL-7R+ cells from the graft (Fig. 4C). The LinSca-1lowIL-7R+ population has been shown to contain CLP, proven using clonal assays to demonstrate both B and T cell potentials in the absence of myeloid potential (18). Depletion of these progenitors by selecting for the Thy1.1 c-KitlowIL-7R+ population eliminated a dramatic peak in B/thymocyte engraftment between days 12 and 17 posttransplant (Fig. 2C). However, this engraftment profile persisted at later time points in 20–30% hosts receiving the c-Kitbright fraction, supporting the conclusions that an early lymphocyte progenitor precedes c-KitlowIL-7R+ CLP stage of development, and that this population of cells resides in the c-Kitbright fraction (26). Furthermore, a lack of IL-7R+ progenitors did not appear to affect combined thymocyte and peripheral B cell engraftment among mice receiving limiting doses of c-Kitbright progenitors (Table IV).

The predisposition of Thy1.1 progenitors to lymphoid development following i.v. transplantation was not reflected by in vitro clonogenic assays. Although the Thy1.1 c-Kitbright population proved highly clonogenic in methylcellulose culture when stimulated with a broad spectrum of cytokines, the colonies that developed were almost entirely myeloid in nature. This could be due to the presence of highly clonogenic myeloid progenitors within the Thy1.1 c-Kitbright compartment that did not proliferate sufficiently in vivo to be detected by our assay. Additionally, the in vitro culture results may suggest that the in vivo lymphoid progenitors present in this population are not yet competent to generate B lineage progeny in response to cytokine stimulation in vitro, and require further nurturing in a specific hemopoietic niche before they are able to expand into lymphoid lineages.

Engraftment patterns that included myeloid lineages were not commonly observed in our analysis. For example, combined B/myeloid and B/T/myeloid engraftment represented only 4 and 3, respectively, of 88 animals transplanted with unseparated Thy1.1 progenitors (Fig. 2C). Nevertheless, selection for the c-Kitbright fraction of Thy1.1 progenitors successfully enriched for myeloid potential, and transplantation of this population resulted in a >7-fold increase in frequency of combined B/T/myeloid engraftment. Bilineage B/myeloid engraftment did not increase in frequency appreciably with c-Kitbright selection; however, this profile became apparent much earlier during engraftment kinetics (Fig. 4B). Simultaneously, transplants with c-Kit-low and -negative fractions did not produce myeloid engraftment (Fig. 3). Several laboratories have observed combined myeloid and B cell potential arising from clonal cultures of primary hemopoietic tissues (4, 20–23, 27). The data presented in this study indicate that segregating the Thy1.1 progenitors on the basis of c-Kit expression allows for the separation of this mixed lymphoid-myeloid potential from those progenitors that produce strictly lymphoid progeny posttransplant. Furthermore, the additional lineage potential found among c-Kitbright progenitors implies that this population is more primitive and may function as a parent population to the Thy1.1 progenitors that have down-regulated c-Kit expression.

Taken together, the data presented in this study support the existence of a novel progenitor within the Thy1.1 c-Kitbright compartment that possesses substantial prothymocyte properties not previously reported from adult mouse bone marrow. The enrichment for progenitors with prothymocyte potential described in this study is restricted to mice expressing both the Ly-6A.2 allele of the Sca-1 Ag and the Thy1.1 allele of CD90. Nevertheless, the existence of such a bone marrow progenitor is probably not restricted to mice with these characteristics. The in vivo nature of these studies has provided a stringent assay for testing these potential progenitors in competition with endogenous hemopoietic recovery. Additionally, this system requires candidate progenitors to independently home to their selected engraftment niches and expand in those sites under as natural of circumstances as could be provided. The bone marrow prothymocyte population described in this study also possesses substantial B lineage potential, and, given their robust in vivo engraftment potential, may represent a novel population of multipotent early lymphoid progenitors in adult mouse bone marrow.

References


