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Phenotypic Distinction and Functional Characterization of Pro-B Cells in Adult Mouse Bone Marrow

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A lymphoid-committed progenitor population was isolated from mouse bone marrow based on the cell surface phenotype Thy-1.1<sup>neg</sup>Sca-1<sup>pos</sup>c-Kit<sup>pos</sup>Lin<sup>neg</sup>. These cells were CD43<sup>pos</sup>CD24<sup>neg</sup> on isolation and proliferated in response to the cytokine combination of steel factor, IL-7, and Flt3 ligand. Lymphoid-committed progenitors could be segregated into more primitive and more differentiated subsets based on expression of AA4.1. The more differentiated subset generated only B lymphoid cells in 92% of total colonies assayed, lacked T lineage potential, and expressed Pax5. These studies have therefore defined and isolated a B lymphoid-committed progenitor population at a developmental stage corresponding to the initial expression of CD45R.


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Materials and Methods

Mouse strains

B6.PL and AKR mice were obtained from The Jackson Laboratory (Bar Harbor, ME), while C57BL/Ka, B6-Thy-1.1-Ly-5.1, and B6-Ly-1.1 mice
were bred and maintained at the Animal Resource Center facility of the University of Utah. Mice used were 4–16 wk of age.

Cytokines and Abs
Steel factor (STL) and G-CSF were gifts from Genmire (San Diego, CA), a subsidiary of Kirin Brewery (Tokyo, Japan). Flt3 ligand (Flt3L) and IL-6 were kindly provided by Immunix (Seattle, WA). Recombinant human erythropoietin (Epo) was purchased from Ortho (Raritan, NJ). Recombinant murine IL-3 and IL-7 were purchased from PeproTech (Rocky Hill, NJ). The cytokines were used at the following concentrations: STL, 100 ng/ml; G-CSF, 10 ng/ml; Flt3L, 75 ng/ml; IL-6, 20 ng/ml; Epo, 5 U/ml; IL-3, 10 ng/ml; and IL-7, 10 ng/ml.

Liquid cultures
CD8, Mac1, Thy-1.1, and IL-7R were conjugated to c-Kit, CD45R, and Gr-1, respectively, by Kappa Scientific (San Mateo, CA). Zymosan (Sigma, St. Louis, MO), and 0.1 mM 2-ME (Mallinckrodt, Chesterfield, MO) supplemented with the indicated cytokine combinations. Culture dishes were incubated at 37°C and infused with 5% CO2. The number of colonies was counted using an inverted microscope after 7 days of culture (Mallinckrodt) and supplemented with the indicated cytokine combinations. Cytokines and Abs were either grown in bulk or seeded at limiting dilution one cell per well) in 96-well plates with or without stromal cell feeder layers as indicated. Culture plates were incubated at 37°C and infused with 5% CO2. The 2018 stromal cell line (a gift from Kateri Moore) was maintained at 31–33°C. Cells were prepared the day before coculture by seeding 10,000 cells/well in 24-well plates for bulk cultures and 1,000 cells/well in 96-well plates for the limited dilution (clonal) assays. The presence of a single cell per well in 96-well plates was confirmed whenever possible after overnight culture using an inverted microscope.

Preparation of BM cells and isolation of hematopoietic stem/progenitor cell populations
The procedure for the preparation of BM cells for sorting has been previously described (20). Briefly, BM cells were isolated from femurs and tibia of donor mice, and the RBCs were lysed in an ammonium chloride potassium solution. The cells were incubated in a lineage cocktail containing optimized concentrations of Abs to CD2, CD3, CD5, Mac-1, Gr-1, TER119, and CD45R. The CD45R Ab was not included in the lineage cocktail whenever CD45R expression was evaluated after lineage depletion. Lineage depletion was conducted by two successive incubations of the BM cells in sheep anti-rat Ig-coupled magnetic beads (Dynal, Oslo, Norway). The Lin- cells were stained with PE-Sca-1 and sorted using the FACSVantage (Becton Dickinson, San Jose, CA) set at enrichment mode and thresholding on PE emissions above background levels. Dead cells were excluded from all analyses and sorts by gating on forward scatter and PI staining. The sorted Lin- Sca-1+ cell populations were pelleted and stained with allophycocyanin-c-Kit and FITC-Thy-1.1 and sorted into Thy-1.1low and Thy-1.1neg cell fractions.

Preparation of thymic lobes
Intrathymic T cell development assay
Sublethally irradiated B6 (4- to 6-wk-old females) mice were anesthetized and immobilized with rubber bands. The skin over the chest was incised to reveal the sterno, which was cut. The thymus was visualized within the thoracic cavity, and 3 μl of fluid containing the sorted cell population of interest was directly injected into the thymic tissue using a Hamilton syringe (Scientific Systems, Westwood, MA). The cells to be transplanted were obtained from the B6-Thy-1.1-Ly-5.1 double-congenic strain and were sorted directly into a microfuge tube containing a known amount of Hanks’ 10% FCS so that each 1 μl of fluid contained a known number of cells. Graded doses of cells were injected into groups of animals (10 animals/group) in the presence of an excess of IL-2. Cells obtained from a second B6 congenic strain (B6-Ly-1.1), which served as a carrier and as an internal control to indicate successful engraftments. Three or 4 wk later, the recipient B6 mice were sacrificed, and thymic tissue was isolated for analysis by flow cytometry to identify thymic lobes containing progeny cells derived from the injected populations. Successful intrathymic transfers were identified by the presence of Ly-1.1+ cells, and positive thymic lobes were scored for the presence of Ly-1.1+ cells. Limiting dilution statistics were applied to the resulting data to derive the frequency of repopulating cells in the sorted population.

RT-PCR assay
Sorted cells were lysed using 500 μl of TRIZol (Life Technologies) with 20 μg of glycogen (Roche, Indianapolis, IN) added as a carrier. The TRIZol protocol for RNA isolation prescribed by the manufacturer was followed using half volumes. After isopropanol precipitation, the RNA pellet was washed twice in 70% ethanol and resuspended in 8 μl of diethylpyrocarboxylate-treated water. The RNA samples were incubated with 1 μl of amplification grade DNase I (Life Technologies) and 1 μl of 10× DNase I buffer at room temperature for 15 min to eliminate any contaminating DNA. The reaction was stopped with the addition of 1 μl of 25 mM EDTA and heating at 72°C for 10 min. Water was added to bring the total volume of each reaction to 20 μl. Five to 10 μl from each total RNA sample was used for first-strand synthesis using random primers (Life Technologies) and Monelone murine leukemia virus reverse transcriptase (Life Technologies) following the protocol provided by the manufacturer.

Semiquantitative PCR was used to compare the expression of genes between sorted cell populations. All primer sequences used in this study have been previously described. To equalize for cDNA input, each sample was first amplified by PCR using GADPH primers (21), and the amount of input cDNA was adjusted to provide equivalent signals. Subsequent PCR amplifications used the predetermined amount of cDNA with gene-specific primers for sterile Ig heavy chain transcript (μs, Rag-2, E2A, Pax-5, and CD19 (7, 22, 23). PCR cycle parameters used for GADPH, μs, and Rag-2 were described by Li et al. (22), while those for E2A, Pax-5, and CD19 were described by Bain et al. (23). Fifteen-microliter aliquots were withdrawn at cycles 24, 27, and 30 (GADPH) or cycles 27, 30, and 33 (μs, Rag-2, E2A, Pax-5, and CD19) that appeared that any PCR product was within the linear range. The PCR products were separated by 1% agarose gel electrophoresis. Quantitation was performed using the MultiAnalyst program (Bio-Rad, Hercules, CA). Individual bands were measured and normalized to GADPH expression for each sample. Comparison of gene expression between samples was achieved by comparing the normalized value for each sample to the value obtained for GADPH by PCR amplification.

Results
The Thy-1.1mum cell population contains three separate progenitor subsets
Lin- mouse BM cells were stained with Abs to Sca-1, c-Kit, and Thy-1.1 and sorted to recover the Thy-1.1low and Thy-1.1mum cell populations (Fig. 1). The Thy-1.1mum subset comprised 0.05 ±

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FIGURE 1. BM cells isolated from adult mice were lineage depleted and subsequently stained with mAbs to c-Kit, Sca-1, and Thy-1.1. Dead cells were excluded using propidium iodide staining and forward scatter gating. Sca-1<sup>low</sup> cells were selected by gating (A) and analyzed for Thy-1.1 expression (B). Thy-1.1<sup>low</sup>Sca-1<sup>low</sup>c-Kit<sup>pos</sup>Lin<sup>neg</sup> (Thy-1.1<sup>low</sup>) and Thy-1.1<sup>low</sup>Sca-1<sup>low</sup>c-Kit<sup>pos</sup>Lin<sup>neg</sup> (Thy-1.1<sup>low</sup>) cells were isolated by cell sorting as described in Materials and Methods, and aliquots were taken for reanalysis (C and D).

Table I. Cloning efficiency and lineage potential of Thy-1.1<sup>low</sup> and Thy-1.1<sup>neg</sup> subsets<sup>a</sup>

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Cytokine Stimulation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Colonies/1000 Cells ± SD&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Lineage Content (% of total, n)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thy-1.1&lt;sup&gt;neg&lt;/sup&gt; progenitors</td>
<td>S7F/6EG</td>
<td>148 ± 44</td>
<td>Lymphoid: 33%, 16</td>
</tr>
<tr>
<td></td>
<td>S7F/6EG (no IL-3)</td>
<td>81 ± 29</td>
<td>Lymphoid: 60%, 12</td>
</tr>
<tr>
<td>Thy-1.1&lt;sup&gt;low&lt;/sup&gt; HSC</td>
<td>S7F</td>
<td>25 ± 1</td>
<td>Lymphoid: 100%, 21</td>
</tr>
<tr>
<td></td>
<td>S7F/6EG</td>
<td>581 ± 71</td>
<td>Lymphoid: 0%, 0</td>
</tr>
<tr>
<td></td>
<td>S7F</td>
<td>88 ± 44</td>
<td>Lymphoid: ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lin<sup>neg</sup> BM cells were sorted to isolate Thy-1.1<sup>low</sup> Sca-1<sup>low</sup> c-Kit<sup>pos</sup> HSC (Thy-1.1<sup>low</sup>) or Thy-1.1<sup>neg</sup> Sca-1<sup>low</sup> c-Kit<sup>pos</sup> progenitor cells (Thy-1.1<sup>neg</sup>) as shown in Fig. 1. Cultures were initiated in methylcellulose medium containing the indicated cytokines as described in Materials and Methods. Cytokines are abbreviated as follows: S, STL; 7, IL-7; F, Flt3L; 3, IL-3; 6, IL-6; E, Epo; G, G-CSF.

<sup>b</sup> Cultures were counted on days 7-9 of culture. Cultures initiated with Thy-1.1<sup>neg</sup> cells stimulated with S7F contained 600 cells/plate; all other cultures were initiated with 120 cells/plate.

<sup>c</sup> To evaluate lineage content, individual colonies were isolated from the methylcellulose medium and split for flow cytometric and histological analysis as described in Materials and Methods. Of all colonies isolated from culture, 70% (Thy-1.1<sup>neg</sup>) to 90% (Thy-1.1<sup>low</sup>) contained enough cells for analysis. The percentage of the total and number of colonies evaluated (n) are presented.

0.01% (mean ± SD; n = 8) of nucleated BM cells, a frequency very similar to that of the Thy-1.1<sup>low</sup> subset as previously reported (1, 2). Virtually all cells expressing low levels of Thy-1.1 were c-Kit(high), while the Thy-1.1<sup>neg</sup> population included cells expressing both low and high levels of c-Kit. Cells lacking c-Kit expression were not further characterized in these studies. Because of concerns regarding contamination of Thy-1.1<sup>neg</sup> cell preparations with Thy-1.1<sup>low</sup> HSC, reanalysis of sorted populations was always performed as shown in Fig. 1, and Thy-1.1<sup>neg</sup> populations containing any discernable contamination with cells expressing Thy-1.1 at a level 5- to 10-fold above background levels were not used for functional studies. Although our previous transplant studies demonstrated an inhibitory influence of allophycocyanin-conjugated c-Kit Abs on in vivo engraftment of Thy-1.1<sup>neg</sup> cells (19), direct comparisons of cloning efficiencies and lineage potentials of Thy-1.1<sup>neg</sup> cells isolated using biotin or allophycocyanin conjugates of anti-c-Kit Abs showed no differences in our in vitro studies.

Initial in vivo transplant studies demonstrated that the Thy-1.1<sup>neg</sup> cell population mediates rapid BM engraftment and contributes to both lymphoid and myeloid lineages (19). To determine whether the Thy-1.1<sup>neg</sup> cell subset consisted of multipotent progenitors or separate progenitor cells committed to either lymphoid or myeloid lineages, we conducted clonal progenitor cell assays. The sorted cells were cultured in methylcellulose or single-cell liquid cultures supplemented with different cytokine combinations, and individual colonies were analyzed after 6–12 days in culture by both flow cytometry and cell morphology. These analyses showed that the Thy-1.1<sup>neg</sup> cell subset contained three types of progenitors (Table I). Stimulation with a mixture of seven cytokines, as detailed in Table I, allowed differentiation of multiple hematopoietic lineages. Under these culture conditions, we observed colonies consisting solely of myeloid lineage cells (macrophages, primitive granulocytes, and erythroid cells), colonies consisting solely of lymphoid lineage cells (CD45R<sup>pos</sup>Gr-1<sup>neg</sup>), and mixed colonies containing both lineages. Lymphoid lineage colonies represented 33% of the total Thy-1.1<sup>neg</sup> colonies analyzed, while those consisting of myeloid lineage cells represented 54% (Table I). Mixed lineage colonies, containing CD45R<sup>pos</sup> cells as well as myeloid cells, were observed at a frequency of 13%. Similar results were obtained in liquid cultures initiated from single cells, suggesting that the mixed lineage colonies were not the result of sampling error in the methylcellulose assay. When Thy-1.1<sup>low</sup> HSC were cultured under the same conditions, pure lymphoid colonies were not observed, and very few mixed lineage colonies containing CD45R<sup>pos</sup> cells were scored (4%, or 2 colonies of 45 examined; Table I). Stimulation of Thy-1.1<sup>low</sup> HSC with lymphoid-specific cytokines (STL, IL-7, and Flt3L) resulted in a marked decrease in cloning efficiency (88 vs 581 colonies/1000 cells plated).

IL-3 has been reported to inhibit early B lymphoid differentiation (24, 25). Consistent with this observation, we observed that omission of IL-3 from the cytokine cocktail resulted in a 40–50% decrease in the cloning efficiency of Thy-1.1<sup>neg</sup> cells. Analysis of the colonies that grew in the absence of IL-3 demonstrated a decrease in the frequencies of pure myeloid and mixed colonies and a concomitant increase in the proportion, but not the absolute number, of pure lymphoid colonies. Comparison between S7F/6EG stimulation with or without IL-3 showed that 33% of 148 ± 44 colonies were lymphoid in the presence of IL-3 (48.8 ± 14 colonies/1000), whereas 60% of 81 ± 29 colonies were lymphoid in the absence of IL-3 (48.6 ± 17 colonies/1000; Table I). Thy-1.1<sup>neg</sup> cells grown in methylcellulose cultures supplemented with cytokines permissive only for lymphoid differentiation (S7F) exhibited a 6-fold decrease in cloning efficiency compared with the more complex cytokine combination, and all colonies evaluated from
these cultures contained only cells with lymphoid morphology and surface Ag expression. The presence of pure lymphoid colonies after stimulation of Thy-1.1\textsuperscript{neg} cells with the complex cytokine mixture confirms that a committed lymphoid progenitor population is contained within the Thy-1.1\textsuperscript{neg} cell subset, but not in the Thy-1.1\textsuperscript{low} HSC population. These cells grow in response to the cytokine combination of S7F and are insensitive to the inhibitory effects of IL-3.

**A number of early B lymphoid markers fractionate the Thy-1.1\textsuperscript{neg} cell population into distinct subsets**

To determine whether additional markers could potentially fractionate the Thy-1.1\textsuperscript{neg} cell population into functionally distinct subsets, we isolated Thy-1.1\textsuperscript{neg} cells and evaluated the expression of a number of cell surface markers known to be expressed during the early stages of lymphoid development. Representative FACS plots of the staining analyses are shown in Fig. 2. As shown in Fig. 2A, the AA4.1 mAb identifies a subset comprising 30–50% of the Thy-1.1\textsuperscript{neg} cells that expresses low levels of c-Kit. Expression of IL-7R also separated the Thy-1.1\textsuperscript{neg} population into two distinct clusters that correlated with c-Kit staining intensities. Cells that were IL-7R\textsuperscript{pos} invariably expressed low levels of c-Kit, while IL-7R\textsuperscript{neg} cells were both c-Kit\textsuperscript{low} and c-Kit\textsuperscript{high} (Fig. 2B). In contrast, CD62L/MEL14 expression was observed on both the c-Kit\textsuperscript{low} and the c-Kit\textsuperscript{high} subsets of Thy-1.1\textsuperscript{neg} cells (Fig. 2C). Of the other cell surface Ags tested, CD4 staining revealed only a minor subpopulation of positive cells (10% of Thy-1.1\textsuperscript{neg} cells, equally distributed between the c-Kit\textsuperscript{low} and c-Kit\textsuperscript{high} subsets, Fig. 2D), which was similar to the distribution of the Sca-2 Ag (data not shown). CD3 served as a negative control and was expressed by <1% of Thy-1.1\textsuperscript{neg} cells (Fig. 2E). Since expression of AA4.1 and IL-7R have previously been associated with early lymphoid progenitors (4, 6, 7, 26), we focused on the c-Kit\textsuperscript{low} subset of cells for additional phenotypic and functional analysis.

A hierarchy of progenitors in the B lymphocyte developmental pathway has been described by Hardy and colleagues, who used cell surface Ag expression to define specific developmental stages (4, 7, 27). To better place the Thy-1.1\textsuperscript{neg} c-Kit\textsuperscript{low} progenitor population in the context of the previous studies, we evaluated CD45R, CD24, and CD43 expression by these cells. Unlike the primitive pro-B cell described by Hardy’s stage A, Thy-1.1\textsuperscript{neg} c-Kit\textsuperscript{low} cells largely lack expression of CD45R and express high levels of CD24 (Fig. 2F, G and H). In common with Hardy’s stage A of development, Thy-1.1\textsuperscript{neg} c-Kit\textsuperscript{low} cells express lower levels of CD43 relative to Thy-1.1\textsuperscript{low} stem cells (Fig. 2H). Hardy and colleagues used expression of AA4.1 along with CD4, CD24, and CD43 to identify a CD45R\textsuperscript{neg} stage of B cell development preceding the A stage (7), but the isolation protocol used in those studies failed to segregate these cells away from erythroid lineage progenitors (4). Furthermore, functional assessment of B lineage precursors in adult mouse bone marrow established that most early B lineage progenitors express CD24, as detected using either the M1/69 or 30-F1 Ab (9). To summarize, Thy-1.1\textsuperscript{neg} c-Kit\textsuperscript{low} cells overlap with Lin\textsuperscript{neg}TdT\textsuperscript{+} cells as defined by Tudor et al. (9), but differ from fraction A\textsubscript{p} as defined by Hardy and colleagues (7) in that most Thy-1.1\textsuperscript{neg} c-Kit\textsuperscript{low} cells lack CD4 and express high levels of CD24 (Fig. 2, F and G).

The lymphoid-committed progenitors in the Thy-1.1\textsuperscript{neg} cell population are predominantly c-Kit\textsuperscript{low}

To address the developmental potential of the individual subsets of cells isolated in the Thy-1.1\textsuperscript{neg} population, we used levels of c-Kit and AA4.1 expression as selection criteria to sort the Thy-1.1\textsuperscript{neg} population into three subsets: c-Kit\textsuperscript{high}AA4.1\textsuperscript{neg}, c-Kit\textsuperscript{low}AA4.1\textsuperscript{neg}, and c-Kit\textsuperscript{low}AA4.1\textsuperscript{pos} (Fig. 2A). Methylcellulose and liquid cultures were initiated with each c-Kit/AA4.1 subset to ascertain their cloning efficiencies and myeloid and/or lymphoid differentiation potential. These cultures were supplemented with cytokine combinations selected to either support proliferation and differentiation toward the lymphoid lineage (STL+ IL-7 + Flt3L, S7F), the myeloid lineage (IL-3 + IL-6 + Epo + G-CSF, S36EG), or both lymphoid and myeloid lineages (STL + IL-7 + Flt3L + IL-3 + IL-6 + Epo + G-CSF, S7F36EG).
Evaluation of the cloning efficiencies of the subsets showed that c-Kit\textsuperscript{high} AA4.1\textsuperscript{neg} cells clonal at a frequency of 20\% in S7F36EG and gave rise to myeloid or mixed colonies, but not pure lymphoid colonies (Table II). Cloning efficiency dropped to 5\% when the cells were stimulated under lymphoid conditions. In contrast, the two c-Kit\textsuperscript{low} populations resolved by AA4.1 staining showed equivalent colony growth in either lymphoid-specific or complex cytokine conditions, suggesting that the majority of clonogenic c-Kit\textsuperscript{low} cells are lymphoid committed (Table II). This interpretation was strengthened by flow cytometric and cytopsin analysis of individual colonies grown in S7F36EG. Of 99 separate c-Kit\textsuperscript{low}AA4.1\textsuperscript{pos} and c-Kit\textsuperscript{low}AA4.1\textsuperscript{neg} colonies analyzed, 88 (89\%) contained only lymphoid lineage cells despite the presence of myeloid-promoting cytokines in the cultures. Mixed lineage colonies were most frequent in the c-Kit\textsuperscript{high} AA4.1\textsuperscript{neg} subset, where they represented 15\% of the total colonies. These progenitors were also identified in both the AA4.1\textsuperscript{pos} and AA4.1\textsuperscript{neg} subsets of c-Kit\textsuperscript{low} cells, with their frequency being higher in the AA4.1\textsuperscript{neg} subset (13\% of total colonies, compared with 6\% among the AA4.1\textsuperscript{pos} cells). Similar results were obtained in single-cell liquid cultures, suggesting that mixed lineage colonies were not due to inadvertent mixing of adjacent single-lineage colonies. Cytopsin analysis confirmed the presence of myeloid cells as detected by flow cytometry. A limited variety of myeloid cells were observed. Neutrophil morphology was limited to very primitive-appearing cells with crescent nuclei. In contrast, mature-appearing macrophages were consistently observed in cytopsin preparations. Purified myeloid colonies arising from Thy-1.1\textsuperscript{neg} c-Kit\textsuperscript{pos} cells were very rare (2\% of total colonies), and colonies containing erythroid lineage cells based on benzidine staining of hemoglobin were never observed in the myeloid or mixed colonies derived from Thy-1.1\textsuperscript{neg} c-Kit\textsuperscript{low} cells. These results confirm previous observations that pro-B cells are characterized by a low level of c-Kit expression (9, 28), and extend these findings by showing that these early pro-B cells can be completely separated from progenitors for nonlymphoid hemopoietic lineages using the Thy-1.1\textsuperscript{neg} selection protocol.

Mixed lineage colonies generated by Thy-1.1\textsuperscript{neg} c-Kit\textsuperscript{high} cells maintained both macrophage and lymphoid progeny over the life of the cultures. In contrast, the mixed lineage colonies detected in cultures initiated with Thy-1.1\textsuperscript{neg} c-Kit\textsuperscript{low} cells (either AA4.1\textsuperscript{pos} or AA4.1\textsuperscript{neg}) were transient, as they could only be detected on day 6 or 7 of culture. A comparison of the growth kinetics of mixed lineage colonies in the c-Kit\textsuperscript{low}AA4.1\textsuperscript{pos} and c-Kit\textsuperscript{low}AA4.1\textsuperscript{neg} cell subsets is shown in Fig. 3A. The c-Kit\textsuperscript{low}AA4.1\textsuperscript{neg} subset did not produce visible colonies until day 7 in culture. At this time, an equivalent number of pure lymphoid and mixed colonies was observed. On subsequent days, only pure lymphoid colonies were detected. A similar pattern was observed when c-Kit\textsuperscript{low} AA4.1\textsuperscript{pos} cells were cultured, except that colonies appeared 1 day earlier, and pure lymphoid colonies always outnumbered mixed colonies. Thus, the mixed lineage potential of the Thy-1.1\textsuperscript{neg} c-Kit\textsuperscript{low} subsets of cells was limited, and detection of the myeloid progeny was transient.

Table II. Cloning efficiency and lineage potential of Thy-1.1\textsuperscript{neg} subsets defined by c-Kit and AA4.1$^a$

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Cytokine Stimulation$^a$</th>
<th>Colonies/1000 Cells $\pm SD$</th>
<th>Lineage Content (% of total, n$^d$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymphoid</td>
</tr>
<tr>
<td>c-Kit\textsuperscript{high} AA4.1\textsuperscript{neg}</td>
<td>S7F36EG</td>
<td>190 $\pm$ 39</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>S7F</td>
<td>50 $\pm$ 25</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>36EG</td>
<td>145 $\pm$ 69</td>
<td>ND</td>
</tr>
<tr>
<td>c-Kit\textsuperscript{low} AA4.1\textsuperscript{neg}</td>
<td>S7F36EG</td>
<td>42 $\pm$ 23</td>
<td>85%</td>
</tr>
<tr>
<td></td>
<td>S7F</td>
<td>53 $\pm$ 26</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>36EG</td>
<td>5 $\pm$ 6</td>
<td>ND</td>
</tr>
<tr>
<td>c-Kit\textsuperscript{low} AA4.1\textsuperscript{pos}</td>
<td>S7F36EG</td>
<td>48 $\pm$ 13</td>
<td>92%</td>
</tr>
<tr>
<td></td>
<td>S7F</td>
<td>50 $\pm$ 29</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>36EG</td>
<td>3 $\pm$ 5</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ Lin\textsuperscript{neg} BM cells were sorted to isolate Thy-1.1\textsuperscript{neg} Sca-1\textsuperscript{pos} c-Kit\textsuperscript{pos} progenitor cells as shown in Fig. 1. This population was further fractionated into the indicated populations as shown in Fig. 2A and 3A.

$^a$ Cytokines were initiated in methylcellulose medium containing the indicated cytokines as described in Materials and Methods. Cytokines are abbreviated as follows: S, STL; 7, IL-7; F, Flt3L; 3, IL-3; 6, IL-6; E, Epo; G, G-CSF.

$^a$ Colonies numbers were evaluated on day 8 of culture.

$^d$ Lineage content was evaluated in Table I on days 6–12 of culture.
The kinetics of colony evolution shown in Fig. 3A suggest that the c-Kitlow AA4.1neg and the c-Kitpos AA4.1pos subsets form a lineage, with c-Kitpos AA4.1neg cells differentiating to generate the more lineage-restricted c-Kitpos AA4.1pos cells. To address this hypothesis, Thy-1.1neg c-Kitlow AA4.1neg cells were grown as replicate bulk cultures in the presence of S7F36EG in 24-well plates. Representative wells were harvested over a period of 8 days, and the cells were stained with mAbs to AA4.1, CD45R, c-Kit, and Gr-1. As shown in Fig. 3B, most of the cells isolated in the Thy-1.1neg c-Kitlow AA4.1neg population become AA4.1pos and CD45Rpos after 5 days in culture. These cultures included rare cells expressing the myeloid lineage marker (Gr-1) on day 5 (data not shown) and day 6, but became predominantly CD45Rpos by day 8. These results, although not at a clonal level, recapitulate the kinetics of CD45R and Gr-1 cell surface expression observed in clonal colonies derived from the c-Kitlow AA4.1neg cells (Fig. 3A). This experiment also provides further evidence that the c-Kitlow AA4.1neg and the c-Kitlow AA4.1pos subsets form a lineage.

It is interesting to note that virtually all cells in these cultures coexpressed AA4.1, CD45R and c-Kit at all time points (Fig. 3B and data not shown). In contrast, parallel cultures initiated with Thy-1.1neg c-Kithigh cells included a heterogeneous pattern of c-Kit expression and few CD45Rpos c-Kitpos cells (data not shown). This result underscores the multilineage potential of the Thy-1.1neg c-Kithigh subset in contrast to the predominantly lymphoid-committed Thy-1.1neg c-Kitlow subsets.

As shown in Fig. 2F, the majority of cells defined by the phenotype Thy-1.1neg c-Kitlow AA4.1pos do not express CD45R, and our clonal assays indicate that this cell subset consists almost exclusively of lymphoid-committed progenitors (Table II and Fig. 3A). However, the efficiency of magnetic bead depletions may not be absolute, particularly for cells expressing low levels of the target Ag. In addition, the cloning efficiency of Thy-1.1neg c-Kitlow AA4.1pos cells (~5%) was equivalent to the low frequency of CD45Rpos cells contained in the Thy-1.1neg c-Kitlow AA4.1pos subset (Table II). To ascertain whether lymphoid lineage potential is indeed contained within the CD45Rneg fraction of the c-Kitlow AA4.1pos cells, we replaced anti-CD45R with anti-CD19 for magnetic lineage depletion and sorted these Linneg cells for CD45Rneg and CD45Rpos fractions within the Thy-1.1neg c-Kitlow AA4.1pos cell subset (Fig. 2F). The cloning efficiencies of both CD45R fractions were comparable (5.8% for CD45Rneg, 4.9% for CD45Rpos), and 96% of colonies assayed (85 of 88) from the CD45Rneg c-Kitlow AA4.1pos cell subset were lymphoid in lineage (data not shown). This result suggests that the Thy-1.1neg c-Kitlow subset represents a developmental stage at which surface expression of CD45R is just beginning to be induced.

To investigate the influence of stromal cell monolayers on the cloning efficiency of the Thy-1.1neg c-Kitlow cell subsets, we established liquid cultures in the presence or the absence of cloned stromal cell lines. Single cells isolated from the AA4.1neg and AA4.1pos subsets of Thy-1.1neg c-Kitlow cells by automatic cell deposition FACS sorting were seeded into microtiter wells containing cytokines alone (S7F36EG or S7F) or in the presence of IL-7 dose-response thresholds leads to preferential outgrowth of pre-B cells expressing productively rearranged IgM in association with the surrogate light chains and signaling components of the pre-B cell receptor (32). These results demonstrate high cloning in 2018 in the presence of S7F had expanded to colonies ranging from 102 to 3 × 104 cells and expressed CD45R, CD24, and BP1 uniformly and CD43 at variable levels. Surface IgM (sIgM) expression was observed on very few cells (data not shown). Bulk cultures established from 5 × 102 AA4.1neg or AA4.1pos cells in S7F with or without 2018 cells expanded to 350- to 900-fold (60–70% viable) in 7 days and 1200-fold (30% viable) in 14 days, demonstrating the extensive proliferative potential of these cells. After 14 days in culture, sIgM expression could be detected on a small subset of cells growing either in cytokines alone or on 2018 cells in the presence of S7F (Fig. 4B). However, only about 10% of the cells in the slgMpos population expressed cytoplasmic IgM on day 11, supporting the interpretation that most of the expanding cells in the cultures represent pre-B cells. This result is also consistent with recent studies suggesting that high levels of IL-7 lead to expansion of slgMpos pre-B cells, and that selective regulation of IL-7 dose-response thresholds leads to preferential outgrowth of pre-B cells expressing productively rearranged IgM in association with the surrogate light chains and signaling components of the pre-B cell receptor (32). These results demonstrate high cloning
efficiency and proliferative potential of the Thy-1.1<sup>neg</sup> c-Kit<sup>low</sup> cell subsets.

The T cell potential of the Thy-1.1<sup>neg</sup> cell population is predominantly found in the c-Kit<sup>low</sup>AA4.1<sup>neg</sup> subset

To assess whether the lymphoid-committed Thy-1.1<sup>neg</sup> subsets have committed to the B lymphocyte lineage or, alternatively, are analogous to the previously reported common lymphoid progenitor (6), T cell progenitor cell assays were performed. Limiting dilution analysis was performed by injecting graded numbers of cells (0–85 c-Kit<sup>low</sup>AA4.1<sup>neg</sup> cells and 0–192 c-Kit<sup>low</sup>AA4.1<sup>pos</sup> cells) into thymic lobes of sublethally irradiated mice and scoring the lobes as positive or negative for T cell development 15–25 days later. To control for the efficiency of thymic injections, we injected the sorted cell subset of interest (isolated from B6 congenic mice carrying the Thy-1.1, Ly-1.2, and Ly-5.1 alleles) along with a saturating dose of Lin<sup>neg</sup> cells derived from a second B6-congenic strain (Thy-1.2, Ly-1.1, Ly-5.2) into B6 mice (Thy-1.2, Ly-1.2, Ly-5.2). Successful intrathymic transfers were identified by the presence of Ly-1.1<sup>pos</sup> cells, and these thymic lobes were scored for the presence of Ly-5.1<sup>pos</sup> cells. This analysis showed that the frequency of T cell progenitors within the c-Kit<sup>low</sup>AA4.1<sup>neg</sup> and c-Kit<sup>low</sup>AA4.1<sup>pos</sup> cell subsets was 1 in 160 and <1 in 620, respectively (Fig. 5). This result supports the interpretation that expression of AA4.1 coincides with B lineage commitment, since a frequency of 1 in 620 could be explained by contamination with populations of cells outside our sorting gates. The low frequency of T cell progenitors in the c-Kit<sup>low</sup>AA4.1<sup>pos</sup> population suggests that common lymphoid progenitors can account for only a subset of these cells.

Expression of B lineage-associated genes in the Thy-1.1<sup>neg</sup> c-Kit<sup>low</sup> lymphoid-committed progenitor subsets

The results of the in vitro clonal assays and the intrathymic injections suggest that the majority of c-Kit<sup>low</sup>AA4.1<sup>pos</sup> cells are committed to the B lymphoid lineage at a developmental stage coincident with up-regulation of CD45R expression. Commitment to the B lymphoid lineage occurs before Ig heavy chain rearrangement (4) and is associated with transcriptional activation of this locus, resulting in the expression of germline μ transcripts (μ<sub>λ</sub>) (7) as well as the expression of a number of genes that are required for Ig gene rearrangements (recombinase-activating gene (Rag)-1 and Rag-2) (22). Transcription of μ<sub>λ</sub> has been shown to reflect the accessibility and competence of the μ region for Ig gene rearrangements (7, 33). In addition, recent studies have established the importance of a number of transcription factors in lymphoid development (34). Three of these proteins, products of the Pax5, E2A, and EBF genes, are essential in both B cell lineage commitment and B cell development (23, 35–37). To better establish the stage of development represented by the two AA4.1 subsets of Thy-1.1<sup>neg</sup> c-Kit<sup>low</sup> cells, we performed gene expression analysis by semiquantitative RT-PCR. We determined the expression of μ<sub>κ</sub>, Rag-2, E2A, Pax5, and CD19 in the various Thy-1.1<sup>neg</sup> cell populations compared with CD45R<sup>pos</sup> BM cells (Fig. 6). Rag-2 expression was detected at equivalent levels in all samples. In contrast, μ<sub>κ</sub> transcripts were highly expressed by Thy-1.1<sup>neg</sup> c-Kit<sup>low</sup> cells and by the two AA4.1 subsets relative to the total population of CD45R<sup>+</sup> cells. Since the target sequence for the 5′ oligonucleotide used to prime this amplification is deleted upon IgH-DJ rearrangement (7, 33), this result demonstrates that both AA4.1 subsets include cells with at least one transcriptionally active allele of the IgH gene in germline configuration. Fig. 6 also provides further evidence supporting the conclusion that AA4.1<sup>pos</sup> cells are the precursors of AA4.1<sup>neg</sup> cells. Although each population equally expressed the E2A gene, both Pax5 and CD19 were up-regulated in AA4.1<sup>pos</sup> cells relative to AA4.1<sup>neg</sup> cells. This observation is consistent with transfection experiments that indicate induction of Pax5 by E2A and of CD19 by Pax5 (38–40). Thus, the Thy1.1<sup>neg</sup> c-Kit<sup>low</sup>AA4.1<sup>neg</sup> stage of development corresponds to the aberrant B cell progenitors in Pax5-deficient mice that can be lineage redirected using IL-7 and other cytokines (36). Together with our functional data, these molecular results strongly support the conclusion that the Thy-1.1<sup>neg</sup> c-Kit<sup>low</sup> subsets include cells at the earliest stages of B cell development in the mouse.

Discussion

Transplant studies have previously shown that the Thy-1.1<sup>neg</sup> population is enriched for committed lymphoid progenitors that can readily engraft in the recipient BM (19). Here we demonstrate that the Thy-1.1<sup>neg</sup> cell population contains three progenitor populations, including separate committed progenitors for both lymphoid
and myeloid lineages as well as a mixed lineage progenitor. Compared with the Thy-1.1low HSC population, Thy-1.1neg cells have a more restricted pattern of myeloid lineage potential because most colonies contained macrophages, while mature neutrophils with segmented nuclei were rare. Moreover, the Thy-1.1neg cell population could be further segregated based on the level of c-Kit and AA4.1 expression. Using these Ags, we delineated two functionally distinct populations: c-Kithigh AA4.1neg cells, which are predominantly committed myeloid progenitors, and c-Kitlow AA4.1neg/pos cells, which are predominantly lymphoid-committed progenitors (Table II). Thy-1.1neg c-Kitlow cells largely share the CD45RPhi phenotype of the fraction A0 cells isolated by Allman et al. (4). However, in contrast to fraction A0, Thy-1.1neg c-Kitlow cells are CD24pos, CD4neg and do not include erythroid lineage progenitors. Tudor and colleagues have shown that the majority of cells comprising fraction A have a low cloning efficiency and thus probably do not represent a major intermediate in B cell development (9). The similarity in phenotype, frequency, cloning efficiency, and differentiation potential of Thy-1.1neg c-Kitlow cells compared with the Linneg c-KitlowIL-7Rag1Flk-2posCD34neg cells described by Tudor and colleagues suggests that the two isolation protocols define a similar population of cells. However, the selection protocol described in the present studies may provide a better separation of lymphoid and myeloid progenitors, since up to 30% of clones grown on S17 stromal cells by Tudor and colleagues were of myeloid lineage. It is likely that the selection against Thy-1.1 expression in the present studies accounts for the increased resolution of B lymphoid progenitors from multipotent stem cells and myeloid progenitors.

Recently, a common lymphoid progenitor with the phenotype Thy-1.1neg Sca-1low c-Kitlow Lin-160 IL-7Rpos has been reported (6). This cell population possesses rapid and prominent lymphoid-restricted potential with limited or no self-renewal activity. Since most of the cells in the Thy-1.1neg c-Kitlow subset express IL-7R (Fig. 2B), this population of cells overlaps almost entirely with the common lymphoid progenitor of Kondo and colleagues. In contrast to the findings of that group, we demonstrate that T lineage potential is low among Thy-1.1neg c-Kitlow AA4.1neg/pos cells and is absent from the Thy-1.1neg c-Kitlow AA4.1neg/pos subset (Fig. 5). One obvious difference between the two sets of experiments is the short-term culture in S7F used by Kondo and colleagues to prove that the clonal progeny of single cells could differentiate into both the T and B lineages. A similar observation was reported by Jacobsen and colleagues, who showed that c-Kithigh Sca-1pos Lin-160 HSC could be cultured for up to 2 weeks in IL-7 and Flt3L and that the cultures, when transplanted i.v., reconstituted T and B, but not myeloid, lineages (41). Older studies by Phillips and colleagues reported similar findings using long term bone marrow cultures transplanted into immunodeficient recipient mice (42). These results are also consistent with studies using animals at the Pax5 locus, since pro-B cells from these animals could be cultured long term in IL-7 while retaining T lineage potential (43). Taken with the results shown in Fig. 5, these observations indicate that common lymphoid progenitors will default to the B lineage unless specific stimulation with cytokines precedes thymic engraftment. This is consistent with an inductive mechanism for the T lineage, rather than a permissive or stochastic developmental pathway.

Kondo and colleagues proposed two pathways of development for conventional B lymphocytes. One of these branches early from the HSCs, has the potential to develop in the T or B lineage but has a low myeloid potential, and expresses IL-7R. The other pathway branches later, has potential to develop as B lineage and a limited array of myeloid cells, and lacks the IL-7R. Although the Thy-1.1neg cell populations reported here as well as that reported by Kondo and colleagues are capable of rapid BM reconstitution, giving rise to predominantly lymphoid lineage cells, the Thy-1.1neg c-Kithigh cell subset as isolated in these studies retains some myeloid differentiation potential. Thus, we suggest that the mixed lineage progenitor described within the Thy-1.1neg c-Kitlow cell subset (Table II) may represent the second branch of B cell progenitors postulated by Kondo and colleagues, while the Thy-1.1neg c-Kitlow AA4.1neg cell population described here may include the common lymphoid progenitors described by Kondo and colleagues. It is not clear to what extent each of these mutually exclusive intermediates contributes to the Thy-1.1neg c-Kitlow AA4.1pos cell subset and subsequent B lineage development (Fig. 7).

Our analysis of T cell potential in the Thy-1.1neg cell population shows that these cells can differentiate in the thymus after intrathymic injection (Fig. 5). Most of the T cell potential is contained in the Thy-1.1neg c-Kithigh AA4.1neg cell subset. However, the frequency of cells in this subset that engraft intrathymically is 32-fold lower than the frequency of T cell progenitors found when Thy-1.1low HSC are injected (2). This suggests that Thy-1.1neg c-Kitlow cells may predominantly be committed to the B cell lineage and that T cell progenitors may largely lie outside this subset of cells. Alternatively, the low frequency of T cell clones observed relative to Thy-1.1low HSC may represent a difference in proliferative expansion before the onset of TCR rearrangements. T cell clones derived from progenitors at later stages of development would appear earlier and would be smaller and may not be observed if they fail positive or negative selection before becoming large enough to be detected in this assay. Most studies indicate that the rate of emigration of progenitor cells into the thymus is quite low (45). Several lines of evidence suggest that it is a committed progenitor rather than a pluripotent HSC that seeds the thymus to initiate T cell development (46–48). We therefore expect to find a BM progenitor with sufficient proliferative activity to maintain production of T lineage cells in the thymus in the absence of large scale replenishment from marrow-derived sources. The sizes of thymic

### FIGURE 7
The developmental relationships between the lymphoid progenitors described in this study and other identified intermediates in early lymphoid development. The HSC has been previously characterized as Thy-1.1neg and negative for a variety of lineage Ags, including CD45R, but positive for c-Kit, Sca-1, CD24, and CD43. Although stem cells in yolk sac and fetal liver express AA4.1, adult BM stem cells do not (44). We have not established to what extent the pro-B cells described in this study are progeny of the common lymphoid progenitors (CLP) described by Kondo et al. (6) or a bipotent B/macrophage progenitor as described by numerous investigators. The T and B lineage potential of the pro-B transitional stage was derived from the frequencies of each activity (1 T progenitor/160 cells vs 1 B progenitor/20 cells). The CLP may be represented by the 10% of T lineage progenitors in the pro-B transitional stage described here, since the phenotypes of these two populations largely overlap.
clones should reflect the amount of proliferation they can undergo before rearrangement of TCR genes and subsequent selection. Additional experiments to localize T cell progenitors immediately outside the HSC compartment will be addressed in the immediate future.

A number of investigators have characterized early B cell development by isolating and characterizing early progenitors based on CD45R expression. The earliest of these progenitors is thought to lack CD24 expression (7, 49, 50) and to up-regulate this marker as differentiation proceeds. The Thy-1.1<sup>neg</sup> c-Kit<sup>low</sup> cell population isolated in these studies has strong hallmarks of being a transitional cell between the HSC and the progenitors for lymphoid lineages. Like the HSC, the Thy-1.1<sup>neg</sup> c-Kit<sup>low</sup> progenitor is CD45<sup>Rneg</sup> and expresses CD24, with the CD24 expression being slightly higher compared with HSC (Fig. 2G). In addition, Thy-1.1<sup>neg</sup> c-Kit<sup>low</sup> cells will proliferate in response to the lymphoid-selective cytokine combination of S7F, exhibiting a cloning efficiency of 4–8% under these conditions (Table II and Fig. 4A).

We observed that cytokine-driven bulk cultures of Thy-1.1<sup>neg</sup> c-Kit<sup>low</sup> AA4.1<sup>pos</sup> cells, in the presence or the absence of stromal cells, supported lymphoid differentiation up to the CD45<sup>Rneg</sup> IgM<sup>neg</sup> B cell stage, but were inefficient at supporting further maturation (Fig. 4B). Modulation of IL-7 concentrations in these cultures may be necessary to select the rare cells that successfully complete Ig gene rearrangement (32, 51).

Recent studies have established the importance of a number of transcription factors in lymphoid development (52). Three of these proteins, products of the Pax5, E2A, and EBF genes, are specifically associated with commitment to the B cell lineage (53–55). Targeted mutation of E2A or EBF results in a block in B cell development at a stage before Ig gene rearrangements, while Pax5-deficient mice initiate D-JH rearrangement but arrest before development at a stage before Ig gene rearrangements, while the combination of SL7 specifically supported lymphoid lineage colonies (Table I). In both cases, however, there was a decrease in cloning efficiency and no marked increase in the number of lymphoid colonies analyzed compared with the complex cytokine combination (S7F36EG). These observations led to the identification of separate committed progenitor cells for lymphoid and myeloid lineages contained within the Thy-1.1<sup>neg</sup> cell population (Table II). The results also imply that the combination of S7F selectively supports the proliferation and differentiation toward the lymphoid lineage of lymphoid-committed progenitor cells, but is not sufficient to influence lineage commitment decisions of mixed lineage progenitor cells. Similar results have been reported for the role of IL-7 in lineage commitment decisions of bipotent lymphoid-myeloid progenitor cells from mouse fetal liver (59). The failure of apoptosis-inhibiting molecules to support continuing differentiation of B lineage cells strongly supports a selective role for extrinsic signals in lymphoid development (60).

Finally, it should be noted that erythroid and megakaryocyte lineage cells were rarely observed in the methylcellulose cultures of Thy-1.1<sup>neg</sup> cells and failed to recover in transplant studies. This observation that the Thy-1.1<sup>neg</sup> cell subset gives rise to lymphocytes, macrophages, and granulocytes, but not erythrocytes and megakaryocytes, provides evidence for the early separation of the myeloid-lymphoid progenitors from the erythroid-megakaryocyte progenitors. Several other laboratories have reported similar findings recently (3, 61–63). This is in contrast to the conventional view of the hierarchy of hemopoietic differentiation, where the lymphoid progenitors are usually depicted as separate from progenitors of the myeloid, megakaryocyte, and erythroid lineages early in the differentiation process. Cell isolation studies such as those reported here will be instrumental in defining the lineage relationships at early stages of hemopoietic development that until this point have remained elusive.

References


