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Primitive Lymphoid Progenitors in Bone Marrow with T Lineage Reconstituting Potential

S. Scott Perry, Robert S. Welner, Taku Kouro, Paul W. Kincade, and Xiao-Hong Sun

Multiple subsets of the bone marrow contain T cell precursors, but it remains unclear which is most likely to replenish the adult thymus. Therefore, RAG-1+ early lymphoid progenitors (RAG-1+ ELP), and CD62L/L-selectin+ progenitors (LSP), as well as common lymphoid progenitors from C57BL6-Thy1.1-RAG-1/GFP mouse bone marrow were directly compared in transplantation assays. The two c-Kithigh populations vigorously regenerated the thymus and were superior to common lymphoid progenitors in magnitude and frequency of thymic reconstitution. Regeneration was much faster than the 22 days described for transplanted stem cells, and RAG-1+ ELP produced small numbers of lymphocytes within 13 days. As previously reported, LSP were biased to a T cell fate, but this was not the case for RAG-1+ ELP. Although RAG-1+ ELP and LSP had reduced myeloid potential, they were both effective progenitors for T lymphocytes and NK cells. The LSP subset overlapped with and included most RAG-1+ ELP and many RAG-1+TdT+ ELP. LSP and RAG-1+ ELP were both present in the peripheral circulation, but RAG-1+ ELP had no exact counterpart among immature thymocytes. The most primitive of thymocytes were similar to Lin-c-KithighL-selectinTdT+RAG-1+ progenitors present in the marrow, suggesting that this population is normally important for sustaining the adult thymus.

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The thymus is remarkable in its ability to produce large numbers of lymphocytes without a resident population of renewing stem cells. Progenitors within the thymus can maintain thymocytopoiesis for only a short period and must be replenished by marrow cells (1). These progenitors are thought to be rare and could be discharged in periodic manner into the blood stream (2). The precise nature of those progenitors remains unclear, and a number of distinct types of bone marrow cells can produce T lineage cells under experimental circumstances (3). This is especially the case when an OP9-Delta-like 1 stromal cell coculture system is used to make T lineage lymphocytes (4–6). Multiple markers, reporters, and gating parameters have been used to resolve those marrow subsets, and it has been difficult to reach consensus about which ones most efficiently colonize the thymus under normal circumstances. We have now performed side by side comparisons of three categories of bone marrow cells with T lymphopoietic potential.

Developmental relationships between multipotent hematopoietic stem cells (HSC) in the marrow and T-lineage committed cells in the thymus have long been investigated (7–9). HSC are known to be present in the circulation and could enter the thymus (10, 11). HSC start producing thymocytes 3 wk after either i.v. or intrathymic transplant (12, 13); however, cells with stem cell properties have not been found in the thymus (8, 11, 14, 15). This observation and the fact that other marrow fractions can more rapidly seed the thymus under experimental conditions suggest that HSC are not directly responsible for maintaining thymocytopoiesis (12, 16). Our focus was on testing marrow cells that rapidly generate large numbers of thymocytes, and on assessing the loss of other differentiation options.

Of the many categories of lymphoid progenitors that have been identified in bone marrow, the most robust thymus repopulating ones have been found among a primitive Lin–c-Kithigh (LSK) multipotent progenitor fraction. Early lymphoid progenitors (ELP) were originally defined as a hormone-sensitive, Flt3/Flk-2+CD27− subset of the Lin+ c-Kithigh compartment (17). They represent the most primitive cells to express lymphoid-restricted genes such as EBF, TdT, RAG-1, or RAG-2 (18). Although ELP are heterogeneous with respect to the combined expression of these genes, RAG-1 represents a useful parameter for sorting viable cells from RAG-1/GFP mice (18). Although not firmly lymphoid committed, they are lymphoid specified and have greatly reduced nonlymphoid differentiation potential when compared with otherwise similar RAG-1− cells. In addition, ELP require much longer intervals to generate CD19+ lymphocytes in culture than Lin–c-Kitlow progenitors (18). Of particular importance, RAG-1+ ELP were more effective and persistent T cell precursors than were c-Kitlow/RAG-1+ prolymphocytes (18).

Consistent with these findings, Adolfsson et al. (19) found effective lymphoid progenitors among LSK with the highest density of Flt3/Flk-2+. When compared with HSC, this fraction had greatly reduced erythroid differentiation potential. Lai et al. (20) found that down-regulation of VCAM-1 on LSK coincided with progression of HSC through the multipotent progenitor stage to become ELP. Cloning and transplantation assays revealed that this transition was accompanied by substantial loss of nonlymphoid differentiation potential.
Long and short term repopulating stem cells in a particular congenic strain of mice express the CD90.1/Thy1.1 marker (12, 21–23). Comparison of progressively smaller bone marrow subsets showed that thymic repopulating capacity was enriched in the CD44+ bone marrow compartment (12). Testing with a panel of Abs revealed that the L-selectin adhesion molecule further defined primitive subsets with lymphoid potential. Intravenous transfer of marrow subsets into sublethally irradiated mice showed that Lin-CD44hiThy1.1 L-selectin+ (L-selectin+ progenitors; LSP) are effective thymus-repopulating cells. LSP defined in this way represent ~17% of the LSK fraction (Ref. 16) and our unpublished observations). Moreover, they are distinctly better T than B cell progenitors (16).

Common lymphoid progenitors (CLP) were originally isolated as Lin-CD44hi-Thy1.1 IL-7Rα+ cells with potential for T, B, and NK cells (23). However, primitive cells are present in the thymus of Ikaros knockout mice that have no CLP, and several studies have shown that primitive thymocytes express low IL-7Rα (14, 24). CLP removed from bone marrow and transferred intrathymically matured faster than cells present in the thymus (23, 24).

Benz and Bleul (25, 26) recently used a chemokine receptor reporter strain to isolate bone marrow cells with T lineage potential. Although some GFP-marked marrow cells were among the LSK subset, most of them were c-Kit+ and expressed IL-7Rα; i.e., they had properties overlapping with those of CLP.

Von Boehmer and colleagues (27, 28) identified more differentiated c-Kit+/H11002 CD45R/B220+CD19-IL-7Rα+ progenitors in pre-Tα reporter transgenics. Although not directly compared with LSK subsets, these CLP2 cells homed to the thymus on transplantation and had B lineage potential.

Clues to the nature of thymus-replenishing cells have also been sought by characterizing primitive progenitors resident within this organ. The most primitive thymocytes lack markers associated with all hematopoietic lineages (Lin−), including CD4 and CD8 (double negative; DN) (29–32). Furthermore, a consensus has developed that these early thymic progenitors (ETP) express high levels of c-Kit (9, 24, 33, 34). The primitive c-Kit+ ETP are uniformly L-selectin+ (16, 35), and regenerating thymocytes in irradiated mice are initially Thy1− (13). As noted here, ETP express low IL-7Rα, but all are Sca1+, and ones that retain some B potential are Flt3/Fk-2− (3, 14, 15, 24, 33, 36). Thymocytes with high levels of CCR9 reporter activity are not T lineage restricted and also produce B, dendritic, and myeloid cells (25, 26). The CD44−CD25− DN1 category can also be divided into five subsets on the basis of CD117 (c-Kit), and CD24 (HSA) expression (14, 24, 33). Two c-Kit+ subsets, designated DN1a and DN1b, were Pot T progenitors, but had little if any B lineage potential (14). Therefore, thymus-colonizing cells would likely be Lin−CD42−/low CD25−Thy1−Scal−c-Kit+Fk-3/Fk-2−CD44−L-selectin− unless they quickly change their characteristics on entry to the thymus. Once arriving, effective T precursors must be capable of rapid and robust expansion to accommodate the rate of lymphocyte production intrinsic to that organ (1, 13, 37, 38).

We have now crossed C57BL/6 (B6)-RAG-1/GFP mice to B6-Thy1.1 animals so that ELP and LSP, as well as CLP, could be isolated and compared. An experimental model was used where transplanted cells home and compete with host progenitors for limited niches in thymuses of sublethally irradiated mice. Although a range of marrow cells have some ability to produce T cells, our findings support reports that most thymic engraftment potential is concentrated among the LSK fraction of bone marrow. Although progenitors previously designated LSP are T vs B lineage biased, they include RAG-1+ ELP that are effective at restoring both cell types. However, none of the primitive ETP category of thymocytes expresses RAG-1, and ELP may replenish the thymus only under circumstances of special need.

### Materials and Methods

#### Mouse

B6 (CD45.2 alloantigen), B6-Thy1.1, BALB/c, and B6-SJL (CD45.1 alloantigen) mice (The Jackson Laboratory) were bred and maintained in the Laboratory Animal Resource Center of the Oklahoma Medical Research Foundation (Oklahoma City, OK). B6-Thy1.1 were crossed with B6-RAG-1/GFP knockin (39) mice to produce animals expressing Thy1.1, RAG-1/GFP, and the CD45.2 alloantigen.

#### Isolation of cell populations and flow cytometry

Cell populations were performed in HBS5 with 5% FCS. Marrow cells were isolated from the long bones of donor mice, and erythrocytes were lysed by briefly resuspending in NH4Cl− hypotonic solution. Progenitors were further enriched by labeling marrow with RB6-8C5 (Ly6G+C), M1/70 (CD11b), TER-119, RM2-5 (CD2), 17A2 (CD3), 53-7.3 (CD5), 53-6.7 (CD8), 1D3 (CD19), RA3-6B2 (B220), and then immunomagnetically depleting cells (Dynatech Laboratories). Because of the possibility of early or passive expression of CD4, this was not used as a lineage depletion parameter. Lineage-negative cells were stained with IL-7Rα PE (PE/SB14), Thy1.1 biotin (Bio; Ox-7; secondary streptavidin PE-Texas Red; Caltag Laboratories), Scal1 PE-Cy5 (D7; eBioscience), c-Kit allophycocyanin (28B8), and L-selectin allophycocyanin-Cy7 (MEL-14; eBioscience), then separated using a FACSÉria flow cytometer (BD Biosciences) into specific populations. Dead cells were excluded by propidium iodide staining (Molecular Probes). Cells harvested were stained with Abs to determine the phenotypes of engrafted cells. Abs included 145-2C11 PE (CD3), GK1.5 PE (CD4), 53-6.7 allophycocyanin, HL3 PE (CD11c), 1D3 allophycocyanin, IAP8 PE (Ly-6G), PK136 allophycocyanin (NK1.1), RA3-6B2 allophycocyanin, AL-21 Bio (Ly6C), and 104 FITC (CD42.5). Analysis was done on a FACSÉalibur (BD Biosciences). For TdT expression, progenitor populations were purified with 5% acetic acid in ethanol and then incubated with rabbit anti-TdT IgG (SuperTechs). GFP cells were illuminated with PE-conjugated goat anti-rabbit IgG (Caltag Laboratories). All Abs came from BD Pharmingen, unless otherwise stated. Purification of each lymphocyte subset was done according to published parameters (16, 18, 23) and was confirmed in each experiment by postsorting analyses (see Fig. 1).

#### Intravenous progenitor transfers

Recipient mice received 6.5 Gy radiation from a 137Cs source (Mark I gamma irradiator; J. L. Shepard and Associates). Mice were anesthetized with isoflurane (Iosol, Vedco), and sorted populations were infused i.v. Host mice were 6- to 12-wk-old B6-CD45.1; donor cells were from 4- to 10-wk-old B6-RAG-1/GFP-Thy1.1 mice expressing the CD45.2 alloantigen. Thymic lobes, spleen, and marrow from one femur were collected separately from recipient mice, and donor-derived cells were assayed by flow cytometry. Limiting dilution analyses of thymic progenitor frequencies were determined by transplanting graded numbers of progenitors to sublethally irradiated mice. At least eight thymic lobes were analyzed at each dose and percentages of thymic lobes containing greater than 105 donor-derived thymocytes at day 17 were determined. Data were pooled from two independent sorting experiments and used to construct lines of best fit (40). Correlation coefficients (r) exceeded 0.9 for each plot.

#### Peripheral blood and DN thymus preparations

Leukocytes were collected from peripheral blood as previously described (41). Lineage-committed cells were identified through incubation with biotin-conjugated lineage Abs, and cell markers were stained as outlined for flow cytometry. Limiting dilution analyses of thymic progenitor frequencies were determined by transplanting graded numbers of progenitors to sublethally irradiated mice. At least eight thymic lobes were analyzed at each dose and percentages of thymic lobes containing greater than 105 donor-derived thymocytes at day 17 were determined. Data were pooled from two independent sorting experiments and used to construct lines of best fit (40). Correlation coefficients (r) exceeded 0.9 for each plot.

#### Fetal thymic organ culture

Fetal thymic organ culture was done as described (42). BALB/c fetuses were harvested at E15, and thymic lobes were cultured in RPMI 1640, 10% FCS with 0.36 mg/ml 2-deoxyglucose for 7 days. Lobes were then transferred to V-bottom culture plates, one lobe per well with 100 progenitors and 200 μl of culture medium. On day 7, medium was replaced with X-VIVO15 (BioWhittaker, Cambrex) supplemented with 1% BSA (StemCell Technologies), 10 ng/ml stem cell factor, and 1 ng/ml IL-7 (R&D

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Results
A model for direct comparison of lymphoid progenitors

Several subsets of bone marrow contain T cell precursors, but it remains unclear which is likely to maintain thymocyteopoiesis (16, 18, 23, 28). We constructed a model for comparison of CLP (Lin\textsuperscript{-}\textsuperscript{c-Kit\textsuperscript{low}Thy1.1\textsuperscript{-}IL7R\textsuperscript{+}}), ELP (Lin\textsuperscript{-}c-Kit\textsuperscript{high}RAG-1\textsuperscript{+}GFP\textsuperscript{+}), and LSP (Lin\textsuperscript{-}c-Kit\textsuperscript{high}Thy1.1\textsuperscript{-}L-selection\textsuperscript{-}). C57BL6 (B6-PL) mice (Thy1.1 allele of CD90) were crossed with B6-RAG-1\textsubscript{GFP}\textsubscript{+/+}\textsubscript{CD90\textsuperscript{-}} mice so that markers would be available to isolate all progenitor populations in parallel (Fig. 1). One thousand sorted progenitors were transplanted i.v. into sub-lethally irradiated (6.5 Gy) CD45 congenic recipients and evaluated for their reconstitution of lineages. Particular attention was paid to the kinetics of thymic colonization, degree of restriction to the T lineage, and numbers of thymocytes produced.

Fig. 2A shows representative CD4 vs CD8 profiles of donor thymocyte recovery over time. All progenitors contributed to thymocyte recovery in advance of the 21 days required by hemopoietic stem cells (12). However, unseparated bone marrow and ELP yielded thymocytes earlier than the other populations.

The degree of lineage restriction and magnitude of thymic engraftment were evaluated in transplanted mice (Fig. 2, B–D). ELP and CLP generated more splenic NK1.1\textsuperscript{-} NK cells than LSP, a trend also in the marrow (Fig. 2B). The kinetics of CD19\textsuperscript{+} B cell production by CLP was 3 days in advance of LSP and ELP (Fig. 2C); however, ELP produced more B cells in the marrow. Compared with transplants of whole bone marrow, the progenitor populations generated only small numbers of myeloid cells (Refs. 18 and 43 and data not shown). Myeloid production peaked on day 16 and was rarely observed thereafter. LSP and ELP expanded at least 20,000-fold in the thymus, whereas CLP expanded 5-fold less (Fig. 2D).

These findings indicated that all three subsets were relatively restricted to lymphoid lineages but differed in the kinetics of thymic recovery. LSP were effective sources of thymocytes, whereas ELP were enriched for T, B, and NK progenitors. CLP peaked early for B lineage differentiation in the marrow and were effective NK producers.

ELP and LSP, but not CLP, produce robust thymic engraftment

The thymus contains primitive cells that repopulate the organ within 9 days of i.v. transplantation (1, 12). A marrow progenitor might have similar kinetics but be capable of more sustained expansion. Therefore, our analysis focused on the speed and durability of T lineage regeneration from marrow subsets (Fig. 3). The kinetics of engraftment was similar in LSP- and ELP-grafted animals, whereas CLP had limited expansion potential (Fig. 3A, left). An early peak was observed in animals transplanted with whole bone marrow, followed by robust replenishment (Fig. 3A, right). This pattern was matched in mice with ELP, whereas LSP-transplanted mice had no early wave, but expansion proceeded from day 15. T lineage cells were generated in CLP-grafted mice from day 16 (data not shown). To determine whether the marrow contained rare progenitors comparable with primitive thymocytes, large numbers (10\textsuperscript{7}) of unfractionated cells were transplanted, but no repopulation was observed before 13 days (Fig. 3A, right). ELP and LSP produced nearly identical numbers of donor thymocytes, and we consider thymic lobes to be engrafted when they contain >10\textsuperscript{5} donor cells (Fig. 3A, left). ELP generated two waves of engraftment within the first 3 weeks posttransplant. Peak activity for ELP in this interval occurred on day 17 (~70%), nearly 2-fold and 7-fold greater than with LSP or CLP, respectively (Fig. 3B). However, LSP engraftment increased with time until the percentages of positive lobes for LSP and ELP recipients were identical. CLP engraftment increased with time but was consistently less than LSP or ELP.

Inspection of these curves suggested that the three subsets contained different frequencies of engraftable thymic progenitors (Fig. 3B), a point addressed with limiting dilution experiments. We calculate that there was at least one progenitor capable of producing detectable engraftment within 17 days of transplant per ~1000 cells in the ELP fraction, as compared with one per ~2000 LSP (Fig. 3C). Low frequencies of engraftment with CLP precluded a similar analysis.

These observations demonstrate that lymphoid progenitors generate an early wave of T lymphopoiesis, followed by a second, more robust engraftment. Whereas the ELP fraction was more enriched with respect to thymus-repopulating cells than LSP, both could restore thymic lobes to normal size within 3 weeks post-transplant. The CLP fraction contributed to T lymphopoiesis but with less efficiency than the other subsets.

The LSP subset contains a majority of the RAG-1\textsuperscript{+} ELP

As shown above, LSP and ELP have similar thymocyte potential after engraftment. We conducted six-color flow cytometry to determine the correlation between these populations (Fig. 4). This revealed that the majority of RAG-1\textsuperscript{+} ELP (Fig. 4A, left) reside in
days at which peak engraftment was achieved per population transplanted), and thymocyte (indicating lineages were evaluated in the indicated tissues. 

CD3, B cell transplanted population. Peak contributions of donor cells to NK (results were seen in experiments that utilized 

lineage potentials of defined progenitor populations. A, Representative plots of thymic engraftment from i.v. transplants of either 

5 x 10^6 whole bone marrow leukocytes (WBM) or 10^3 sorted progenitors. The far left panels show how total thymocytes recover by 13 days (d) posttransplant. The remaining dot plots are gated on donor-type cells and show how transplanted progenitors generate thymocytes over time. Similar results were seen in experiments that utilized >80 thymic lobes for each transplanted population. Peak contributions of donor cells to NK (B), B cell (C), and thymocyte (D) lineages were evaluated in the indicated tissues. Days at which peak engraftment was achieved per population transplanted are noted in parentheses above each bar. Averages were taken from five sorting experiments for LSP, 5 for ELP, and 3 for CLP. Bars, SEM.

the Thy1.1^- subset (>80%, left of gray line). Furthermore, evaluation of Thy1.1 and L-selectin expression on ELPs showed that ~60% are within the previously designated LSP category (Fig. 4A, right). Gating LSPs for RAG-1/GFP expression showed that only 8% of LSP express RAG-1 (Fig. 4A, right). Therefore, many RAG-1^- ELP overlap with but still represent a small fraction of LSP.

LSP failed to generate a peak of thymocytes on day 13 posttransplant, but many RAG-1^- ELP are included in the LSP subset. An explanation could be the low frequency of RAG-1^- ELP in this fraction (Fig. 4A, right). Therefore, we transplanted increasing numbers of LSP and evaluated the frequencies of engrafted thymic lobes on day 13 (Fig. 4B). Engraftment was detectable with LSP when transplant doses exceeded 6 x 10^3 cells (which would include ~500 RAG-1^-). Thus, rare RAG-1-expressing cells in the LSP category could account for a very small early wave of T lymphopoiesis.

These observations raised the question of what T lineage potential in the LSP fraction corresponds to RAG-1^- progenitors. Therefore, we sorted and transplanted several doses of RAG-1/-GFP-negative LSP (RAG-1^- LSP) and evaluated thymocyte production (Fig. 4C). By day 17, 2,000 RAG-1^- LSP produced thymic engraftment comparable with that of 1000 whole LSP; by day 27, 800 RAG-1^- LSP were comparable in thymocyte potential with unseparated LSP and ELP. These results suggest that the early thymic potential of LSP may be a function of the RAG-1^- cells. Even so, the LSP subset includes engrafting thymic progenitors apart from the RAG-1^- fraction. We also found that RAG-1^- LSP are more T lineage restricted than the LSP population as a whole (data not shown).

Cells with ELP and LSP characteristics in blood and thymus

Marrow cells that colonize the thymus must traverse the blood stream. To determine whether LSP or ELP were present in the circulation, we analyzed peripheral blood leukocytes for expression of surface Ags. Analysis for L-selectin and RAG-1/GFP in Lin^-Thy1.1^- peripheral blood showed a fraction of ELP and LSP in the circulation (Fig. 5A). The ratio of LSP to ELP was reduced relative to the marrow (~3:1 in blood vs ~12:1 in marrow). In contrast to a previous report (13), we found CLP in the circulation (Fig. 5A, far right panel) and ~85% of them were RAG-1/GFP^- (data not shown).
ELP were originally defined on the basis of lymphoid gene expression, including TdT (18). Analysis of the primitive c-Kit<sup>high</sup> DN1 fraction of thymocytes revealed uniform expression of TdT (Fig. 5B). The c-Kit<sup>high</sup> DN1 thymus subset also expresses L-selectin (16), so we suspected that TdT might be indicative of thymic progenitors within the LSP subset. Stained in separate experiments, one-half of ELP and one-fourth of LSP expressed TdT. For comparison, only 4% of the common myeloid progenitor subset expressed TdT (Fig. 5C). These results indicate that some markers associated with the most primitive of thymocyte precursors are expressed in marrow cells. The DN1 fraction of the thymus includes cells with LSP characteristics (16). We next asked whether RAG-1<sup>+</sup> ELP overlapped with these progenitors. Fig. 5D shows RAG-1/GFP expression in the DN subsets. All c-Kit<sup>high</sup> DN1 thymocytes were RAG-1/GFP<sup>+</sup>, and only this fraction generated thymocytes in fetal thymic organ culture (Fig. 5E). Therefore, cells phenotypically similar to ELP and LSP were found in the circulation, but L-selectin<sup>+</sup>TdT<sup>+</sup> primitive thymocytes did not include RAG-1<sup>+</sup> cells.

**Discussion**

A major objective of these experiments was to perform a side by side comparison of three categories of lymphoid progenitors. Early and sustained thymic reconstitution was obtained with LSP and RAG-1<sup>+</sup> ELP subsets of the LSP fraction of bone marrow. Flow cytometry analysis revealed a substantial degree of overlap between these two populations. Although purified according to their original descriptions, both of these subsets were heterogeneous with respect to other parameters, and only some of them had characteristics similar to those of ETP. Consistent with previous studies (18, 24), Lin<sup>−</sup>c-Kit<sup>−</sup>IL-7R<sup>α</sup> CLP were much less effective T lineage precursors when assessed by i.v. transplantation.

Although a wealth of information has been obtained by many laboratories, the nature of marrow cells that replenish the adult thymus under normal circumstances remains unclear (3). This could be an infrequent event, and it almost certainly involves very few cells. The issue is further complicated by the fact that many marrow fractions have T lineage potential under experimental conditions. Rigorous comparisons of previously defined progenitors could be helpful, especially when information is available about their relative potency, abundance in the bone marrow, and presence in the circulation.

Spangrude and colleagues (16) and Wu et al. (35) described candidate T lineage progenitors among the Thy1.1<sup>+</sup> subset of the LSK fraction of bone marrow that expressed the CD62L/L-selectin adhesion molecule. These LSP comprise ~17% of the LSK fraction, and we have confirmed that they are better T progenitors than B progenitors in transplanted mice. We estimate that LSP are 12-fold more numerous than RAG-1<sup>+</sup> ELP and 4 times more abundant than CLP. As in a previous study (16), we found that LSP contributed to T lymphopoiesis within 13 days, 9 days faster than CLP. In a previous study, we determined that 60% of RAG-1<sup>+</sup> ELP express L-selectin and lack Thy1.1; i.e., they would be sorted as LSP. However, RAG-1<sup>+</sup> ELP comprise only 8% of the total LSP subset. Approximately 23% of LSP were TdT<sup>+</sup>, another characteristic used to distinguish ELP (17). Overlaps between LSP/ELP subsets are depicted in Fig. 6.

Serial examination of mice transplanted with these populations revealed that RAG-1<sup>+</sup> ELP accounted for the first wave of T cell production (day 13; Figs. 3B and Fig. 4, B and C). This very small peak was followed 1 week later by robust thymic regeneration, when LSP and RAG-1<sup>+</sup> ELP made equivalent contributions. ELP and LSP both generated large numbers of thymocytes, but neither are stem cells because T lymphopoiesis declined 21 days after transplantation. Limiting dilution analyses performed 17 days after transplantation indicated that active T cell progenitors were ~2-fold more enriched in the RAG-1<sup>+</sup> ELP subset than in the LSP fraction. Considering the 2-fold higher percentages of successfully engrafted lobes on day 17 (Fig. 3C), RAG-1<sup>+</sup> ELP may have been superior with respect to thymic homing.

Elegant single cell studies performed by Kondo et al. (23) revealed that CLP have high T potential when injected into the thymus. This was confirmed in another study where CD93<sup>+</sup>A4.1 was used as a gating parameter for CLP (24). CD93<sup>+</sup> CLP produced

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**Figure 4.** Overlap between ELP and LSP populations. Six-color flow cytometric analysis was used to determine the degree of coincidence between ELP and LSP marrow fractions. A, left, 60% of RAG-1<sup>+</sup> ELP express L-selectin and lack Thy1.1, whereas an additional 13% of these progenitors display low levels of Thy1.1 (right box). Right, 8% of LSP (large box) express RAG-1/GFP (small box). The percentages represent means ± SEM from six independent experiments. B, Increasing numbers of progenitors were transplanted to assess competency for rapid regeneration. Thymic lobes containing at least 10<sup>5</sup> donor thymocytes 13 days post i.v. transplant of ELP (large box) and LSP (small box) are shown. Numbers in parentheses represent two independent experiments; the smaller number of each figure indicates the number of animals transplanted. C, Day 13 thymic engraftment seen in LSP and ELP (open and gray bars, respectively) are given for reference.
lymphocytes as early as Flt3/Flk-2/LSK after intrathymic injection but lost activity more rapidly (24). Therefore, we were surprised that T lymphopoiesis was slow and gradual following i.v. transplantation of CLP (Figs. 2D and 3A). It may be that these cells are inefficient with respect to thymic homing and, as suggested by others (3, 23, 24, 44), would normally differentiate to B and NK lineages if left in the marrow.

Primitive hemopoietic cells express low levels of transcripts corresponding to multiple lineages (45, 46), and there is no evidence that lymphoid genes are activated in a synchronous manner. Whereas some transcription factors are essential for initiating the process, patterns of cell surface marker expression are variable and a precise sequence need not be followed to produce lymphocytes of a given type (7, 47, 48). Early lymphoid cells sorted according to selected markers are heterogeneous with respect to others, and we have documented many combinations of CD62L, TdT, RAG-1, and a human μ transgene in individual cells (17, 18). It remains unclear whether acquisition or loss of any marker corresponds to lymphoid lineage specification. Mice transplanted with RAG-1/LSP produced early (day 13) and later (day 20) peaks of thymocytes. Large numbers (up to 64 lobes) of recipient thymuses were evaluated, and the phenomenon was consistent. This could reflect heterogeneity in the populations and may also indicate that some of the cells colonize other hemopoietic organs in sublethally irradiated recipients before seeding the thymus (49).

The degree of lymphoid lineage restriction of progenitors has been controversial and may be assay dependent. For example, CLP produce no myeloid cells in clonal assays driven by recombinant cytokines but do so when placed in stromal cell cocultures (23, 43). This has also been our experience, and we observed small numbers of GR-1+ cells in marrow of CLP recipients soon after

**FIGURE 5.** Characteristics of progenitors in blood and thymus. A, A flow cytometry analysis is shown for Lin−Thy1.1− cells present in peripheral blood. The left panel resolves LSK (R1) and c-Kit−Sca-1− (R2) subsets. The R1 population is further separated according to L-selectin and RAG-1/GFP in the middle panel to show RAG-1+ ELPS and L-selectin−LSP. In addition, c-Kit+IL-7Ra− CLP are gated from the R2 subset and illustrated in the far right panel. B, DN1 thymocytes prepared as described in Materials and Methods were analyzed with respect to c-Kit density and intracellular TdT. C, In two separate experiments, purified bone marrow progenitors were stained for TdT. Average frequencies of TdT+ cells relative to negative controls are shown. D, The thymus DN subsets are resolved in the dot plot and used to set gates for analysis of RAG-1/GFP expression (histograms). E, RAG-1/GFP is not expressed by DN1 thymocytes with high levels of c-Kit (left). The two gated DN1 subsets were placed in fetal thymic organ cultures, and only the c-Kit+ subsets (R3) generated double positive T lineage lymphocytes (middle and right).
transplantation (<day 13; data not shown). It is possible that cells sorted in our laboratory were contaminated with myeloid progenitors, but we used all four of the sort criteria originally described by Kondo et al., and similar results were obtained when CLP were sorted twice. The time after engraftment (before or after 4 wk) and tissues examined (blood vs marrow) represent additional variables between studies. In any case, myeloid potential is lost in a progressive rather than abrupt manner, given that multipotent progenitors acquire LSP or ELP and then CLP characteristics (50, 51).

The marrow cells we studied were sources of B and NK cells, and our experiments do not reveal when T progenitors completely lose those options (Fig. 2). However, there was notable bias associated with the subsets. LSP were particularly effective for producing T cells, whereas recipients of RAG-1 ELP made all lymphoid cell types. Our findings suggest that segregation of cell fates starts at an extremely early stage in bone marrow. There is some evidence for this in lymphoid progenitors present in the fetus (52, 53). As a possible indication of maturity, B lineage lymphocytes emerged in the marrow of CLP recipients faster (<12 days) than in mice transplanted with LSP or ELP (14 days). CLP2 were not included in our study, and it would be useful to assess their potency as T cell progenitors relative to the other subsets (27, 28).

As noted in the Introduction, Lin− Sca1− c-Kithigh CD62L+ CD44high cells resident in the thymus are potent T progenitors and produce lymphocytes within 9 days of transplantation (14, 33, 54, 55). This pattern of surface marker expression corresponds to the characteristics of marrow LSP and ELP. Flt3/Flk-2 is expressed on 15% of whole bone marrow. Gating and resolution of progenitor populations was performed as illustrated in Fig. 4A. The relative incidences among total marrow nucleated cells are depicted with circles of proportional size. The degree that subsets have overlapping properties is also indicated. WBM, whole bone marrow leukocytes.

It has been previously demonstrated that stem cells and progenitors with LSK characteristics are in the circulation (56, 57). This includes cells that express a RAG-2 BAC transgene. We now report that LSP, RAG-1 ELP, and CLP are detectable in the blood. Resolution of the CLP subset was done with the original Kondo gating parameters and may explain a small discrepancy with a previous study (57).

Our analysis was not designed to establish developmental relationships between HSC and lymphoid progenitors present in marrow, blood, and thymus. However, the kinetics of thymocyte production and patterns of marker expression would be consistent with the presence of LSP and ELP immediately downstream of HSC. Although it is difficult to define rare cells that infrequently replenish the thymus, we have characterized defined categories of marrow progenitors and compared them for T lineage potential. It seems probable that some specification for T lymphopoiesis begins in the marrow, but loss of other differentiation options is clearly a gradual process.

Since completion of these experiments, Hardy and colleagues (58) compared marrow progenitors separated according to different parameters. Although not as lymphoid restricted as RAG-1 ELP, a CD24+CD93+ subset of Lin+ c-Kithigh bone marrow cells was superior to CLP and later fractions with respect to T lineage reconstitution.

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References


