L-Selectin Defines a Bone Marrow Analogue to the Thymic Early T-Lineage Progenitor

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Running Title: Bone marrow early T-lineage progenitor
Scientific Heading: Hematopoiesis
Word Counts Total Text: 4970; Abstract: 142

Financial Support
This work was funded by grants from the National Institutes of Health (T32 DK07115 and RO1 DK57899), and the Brian Rooney Fund of the Lymphoma Foundation. S.T. is a research scholar of the American Cancer Society.
Abstract

The recent description of an early T-lineage progenitor (ETP) population in adult mouse thymus implies the presence of a bone marrow predecessor that has not yet been identified. Here we describe a Lin^Neg Sca-1^Pos c-kit^Hi Thy-1.1^Neg L-selectin^Pos adult mouse bone marrow population that resembles the thymic ETP in both antigen expression phenotype and post-transplant lineage potential. These cells produce wave-like kinetics of thymic seeding, and reconstitute the irradiated thymus with kinetics comparable to a thymocyte graft after IV transplant. Transient B lineage reconstitution is also observed, but little myeloid potential can be detected in transplant experiments. A second subset of progenitors is L-selectin^Neg and is highly enriched for rapid and persistent T and B lineage potential, as well as some myeloid potential. L-selectin (CD62L) is therefore an effective marker for separating lymphoid progenitors from myeloid progenitors and HSC in mouse bone marrow.
Introduction

The identity of T and B lymphoid progenitors in the hematopoietic environment of the bone marrow has been a topic of intense investigation for many years. Of particular interest has been the question of whether commitment to the T lineage occurs prior to or after homing of bone marrow-derived cells to the thymus. The direct approach to addressing this question has been to isolate progenitor populations from bone marrow and to evaluate their lineage potential in culture and transplant experiments. Using this approach, several groups have identified bone marrow progenitor cell subsets which are capable of both T and B lineage development, but lack significant levels of myeloid progenitor activity (common lymphoid progenitor, CLP). Precursor cells committed to T lineage differentiation (pre-T cells) have been identified in the thymus and fetal blood but not the bone marrow, suggesting that during adult life progenitor cells capable of both T and B lineage development commit to the T lineage only after entry into the thymus. This interpretation is supported by analysis of primitive mouse thymocyte populations, which has demonstrated the presence of intrathymic progenitors with T, B, NK, myeloid, and dendritic cell potential. In human studies, common progenitors for the T and NK lineages have been identified, and the role of basic helix-loop-helix factors in regulating early human T cell development has been reported. Recovery and re-transplantation of early thymic migrants supports the conclusion that while many cell types initially enter an irradiated thymus, commitment to lymphoid development occurs relatively rapidly. A recently described thymic population, termed the early T-lineage progenitor (ETP), is consistent with the concept of multipotent but lymphoid-biased progenitor cells as the most primitive cells in the thymus. However, the thymic ETP arises independently of previously-described CLP cells in the bone marrow, suggesting that additional pathways of T lymphoid development may exist in the bone marrow. If multiple pathways for T lineage development do indeed exist, it is of considerable interest to determine the degree to which each pathway contributes to the mature T cell pool.
Considerations for such an analysis must include thymic homing, intrathyemic expansion, and degree of contribution to peripheral T cell pools.

We have recently described isolation of an adult mouse bone marrow progenitor population that is capable of repopulating an irradiated thymus within two weeks following IV transfer. Levels of donor cell engraftment were equivalent to those seen after hematopoietic stem cell (HSC) transplant, but reached peak levels nine days more rapidly and persisted for at least 2 months. These cells are identified phenotypically as Lin$^{Neg}$ Sca-1$^{Pos}$ c-kit$^{Hi}$ Thy-1.1$^{Neg}$ (hereafter referred to as the Thy-1.1$^{Neg}$ population). In addition to T lineage potential, the Thy-1.1$^{Neg}$ population harbors notable B lymphocyte but very limited myeloid potential in IV transplant experiments. In the absence of single-cell studies, which are difficult to perform in an IV transplant model, it is unknown whether the Thy-1.1$^{Neg}$ population includes a mixture of separate precursors for T, B, and myeloid lineages, or if the separate lineages derive from multipotent progenitors that are biased toward lymphoid differentiation. Addressing the clonality question in culture models introduces several concerns, including the loss of a requirement for thymic homing and the possibility that lineage commitment may be redirected by culture conditions.

We hypothesize that the Thy-1.1$^{Neg}$ population includes a subset comprising the immediate bone marrow precursor of the thymic ETP. In order to determine if such a progenitor exists within the Thy-1.1$^{Neg}$ population, we screened Thy-1.1$^{Neg}$ cells for markers that are also expressed by primitive thymocytes. We found that separation of the Thy-1.1$^{Neg}$ population based on L-selectin (CD62L) expression resolved two significant populations. The smaller L-selectin$^{Neg}$ fraction harbors a very high degree of B lineage potential, along with high T cell potential and notable myeloid potential. In contrast, the L-selectin$^{Pos}$ fraction contains robust T-lineage progenitor activity, with minor B lineage and myeloid potential similar to the thymic ETP. Furthermore, in vivo analysis showed that Thy-1.1$^{Neg}$ L-selectin$^{Pos}$ progenitors colonize the thymus with wave-like kinetics reminiscent of normal thymic seeding. Accordingly, we
conclude that the L-selectin<sup>Pos</sup> population includes a bone marrow analogue to the thymic ETP, and likely includes the population from which the thymic ETP originates.

**Materials and Methods**

**Mouse strains**

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

**Antibodies**

Monoclonal antibodies against CD8 (53-6.7), CD11b (M1/70), erythrocytes (TER119), Ly-6G (RB6-8C5), CD3 (KT3-1.1), CD5 (53-7.3), CD2 (Rm2.2), CD45R (B220; RA3-6B2), Thy-1.1 (19XE5), CD19 (1D3), L-selectin (Mel-14), and Ly-5.1 (A20) were purified from media of cultured hybridoma cell lines and were conjugated with biotin, phycoerythrin (PE) or fluorescein (F) in our laboratory. Biotinylated antibodies were secondarily stained with either PE-streptavidin (PE-SAv, Biomeda, Foster City, CA) or F-SAv (Biomeda). PE-conjugated monoclonal antibodies to Sca-1 and CD19, and allophycocyanin-conjugated c-kit (APC-c-kit) antibody were purchased from Pharmingen, Inc. (San Diego, CA).

**Isolation of hematopoietic stem and progenitor cell populations**

Bone marrow cells were isolated as previously described<sup>20</sup> and incubated in a cocktail of antibodies to CD2, CD3, CD5, CD8, CD11b, Ly-6G, TER119, CD45R, and CD19. Lineage depletion was carried out by two successive incubations in sheep anti-rat Ig-coupled magnetic beads (Dynal AS, Oslo, Norway). Lin<sup>Neg</sup> cells were stained with PE-Sca-1 and sorted using a FACS Vantage (Becton Dickinson Immunocytometry Systems, San Jose, CA) in enrichment mode to collect all cells with PE emission above background, excluding dead cells based on forward scatter and propidium iodide (PI; Molecular Probes, Eugene, OR) staining. The sorted Lin<sup>Neg</sup> Sca-1<sup>Pos</sup> cells were stained and resorted in normal mode into specific populations as noted.
in the results. For all experiments, cells were sorted directly onto 20 µL of 100% fetal calf serum (FCS; Hyclone Laboratories, Logan, Utah) contained in the wells of a round-bottomed 96 well microtiter plate.

**Methylcellulose Assays**

Cell populations were cultured in methylcellulose at a plating density of approximately 50 cells per 35 mm culture dish. Each mL of culture media contained alpha-MEM (Gibco BRL, Gaithersburg, MD), 1.2% methylcellulose (Shinetsu Corp., Tokyo, Japan), 30% FCS, 1% deionized bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 0.1 mM 2-mercaptoethanol (Mallinckrodt Chemical Inc., Chesterfield, MO) supplemented with optimized concentrations of the cytokines listed below. Colonies were counted using an inverted microscope, and four to eight plates were scored for each population tested. Individual colonies were plucked from the methylcellulose culture media with a 200 µL pipette, washed in Hanks balanced salt solution with 5% FCS (H5F), and labeled with F-labeled anti-Ly-6G and PE-labeled CD19 antibodies.

**Cytokines**

Steel factor (SF) and granulocyte colony-stimulating factor (G-CSF) were a kind gift from Gemini Science Inc. (San Diego, CA), a subsidiary of Kirin Pharmaceuticals. Flt3 ligand (Flt3L) and interleukin (IL)-6 were kindly provided by Immunex Corp. (Seattle, WA). Recombinant human erythropoietin (EPO) was purchased from Ortho Pharm. Corp. (Raritan, NJ). Recombinant murine IL-3 and IL-7 were purchased from Peprotech Inc. (Rocky Hill, NJ). The cytokines were used at the following concentrations: SF, 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.

**In vivo assessment of progenitor potential**

All recipient mice were exposed to a single 6.5 Gy dose of radiation from a $^{137}$Cs source (Mark I gamma irradiator; J.L. Shepherd and Associates, Glendale, CA). Several hours later, mice were anesthetized with isofluorane (Isosol; Vedco, Inc., St. Joseph, MO) and the purified cell population was infused IV via the retroorbital sinus. Host animals were 6- to 24-week-old
B6 mice, while donor cells were obtained from 4- to 8- week-old B6-Thy-1.1-Ly-5.1 mice. Recipient animals were maintained on oral neomycin sulphate (Biosol, 2 mg/mL; Upjohn, Kalamazoo, MI) for two weeks after irradiation and transplant. At the time points indicated, the recipient B6 mice were sacrificed and each thymic lobe and the marrow from one femur were collected separately. Spleens were also collected in experiments where indicated in the results. Donor-derived cells were identified and phenotyped by flow cytometry using a FACScan analyzer (Becton Dickinson).

To assess the ability of cell populations to rescue lethally irradiated mice, host mice were given a second 6.5 Gy radiation dose three hours after the first dose and prior to IV injection of the purified cells to be tested.

Results

L-Selectin is expressed by bone marrow progenitors and primitive thymocytes

Previous studies have indicated that thymocyte progenitor activity in mouse bone marrow is confined to the Sca-1\textsuperscript{Pos} Lin\textsuperscript{Neg} compartment.\textsuperscript{14} Prothymocyte potential can be further enriched from marrow by selecting for bright expression of CD117 (c-kit\textsuperscript{Hi}),\textsuperscript{21} while negative selection for Thy-1.1 (Thy-1.1\textsuperscript{Neg}) excludes HSC.\textsuperscript{22} Lin\textsuperscript{Neg} Sca-1\textsuperscript{Pos} c-kit\textsuperscript{Hi} Thy-1.1\textsuperscript{Neg} cells largely lack CD127 (IL-7R\alpha), out-compete endogenous thymocyte recovery in mildly-irradiated mice, and repopulate the thymus after IV transfer with kinetics more than one week faster than parallel grafts specifically enriched for HSC.\textsuperscript{14} While the Thy-1.1\textsuperscript{Neg} population exhibits rapid and robust thymic engraftment, it also contributes to B cell and myeloid lineages. In order to further define the prothymocyte potential within this population, we tested other markers for their ability to segregate specific hematopoietic potentials. Interestingly, the adhesion molecule L-selectin (CD62L), which is expressed by both primitive thymocytes\textsuperscript{23} and early bone marrow hematopoietic progenitors,\textsuperscript{24-26} is also expressed by a majority of cells in both the Thy-1.1\textsuperscript{Neg} progenitor population and the Thy-1.1\textsuperscript{Lo} HSC-containing fraction (Figure 1A, 1B). We found
Figure 1: Expression of L-selectin among primitive bone marrow and thymic progenitors. Representative expression of L-selectin and c-kit on Lin$^{Neg}$ Sca-1$^{Pos}$ mouse bone marrow (Panels A and B) and CD4$^{Neg}$ CD8$^{Neg}$ thymus cells (Panel C). For bone marrow statistics, percentages were averaged from nine separate sorting experiments representing marrow pooled from 68 total donor mice. Thymic statistics represent nine separate mouse thymuses. In all cases, error indicates SEM.

that 36±5% of Thy-1.1$^{Neg}$ progenitors are positive for L-selectin expression (L-selectin$^{Pos}$), while 8±1% of Thy-1.1$^{Neg}$ progenitors do not express this marker (L-selectin$^{Neg}$; Figure 1A). Likewise, 43±5% of Lin$^{Neg}$ Sca-1$^{Pos}$ c-kit$^{Hi}$ Thy-1.1$^{Lo}$ progenitors are positive for L-selectin expression and 32±3% are negative (Figure 1B). We also assessed mouse thymocytes lacking CD4 and CD8 (DN thymocytes) for expression of c-kit and L-selectin. This analysis revealed that essentially all DN thymocytes expressing c-kit at high levels also express L-selectin (Figure 1C). Since thymic ETP represent a subset of c-kit$^{Hi}$ thymocytes, we conclude that thymic ETP are also L-selectin$^{Pos}$. Totaled, the c-kit$^{Hi}$ L-selectin$^{Pos}$ fraction represents 6.6±0.7% of DN thymocytes, while c-kit$^{Hi}$ L-selectin$^{Neg}$ compartment comprises less than 0.5%.
L-selectin expression separates hematopoietic populations with distinct thymic engraftment properties

To determine if T lineage progenitors are enriched in either of the subsets of Thy-1.1\textsuperscript{Neg} cells defined by L-selectin as shown in Figure 1A, we conducted a series of transplant experiments into sub-lethally irradiated mice using CD45 allelic variants to enable specific tracking of donor-derived engraftment.\textsuperscript{27} Lineage reconstitution mediated by the progenitor populations was assessed in the 8 weeks following IV transplantation. Thymic lobes and the marrow of one femur were probed for donor-derived cells, using a threshold of $10^5$ donor-derived cells to define positive engraftment. Analysis of thymic reconstitution revealed that both L-selectin fractions were capable of repopulating the thymus (Figure 2A). Of the two subsets, L-selectin\textsuperscript{Pos} progenitors mediated slightly faster thymic engraftment kinetics, generating an average of more than $10^7$ thymocytes per engrafted thymic lobe within 21 days post transplant. However, $10^3$ L-selectin\textsuperscript{Pos} progenitors was not a saturating cell dose at early time points, and on day 21, only 7 of 16 (44%) thymic lobes assayed were positively engrafted (Figure 2B). Parallel transplants with the L-selectin\textsuperscript{Neg} population produced a somewhat higher engraftment frequency, with 75% of thymic lobes engrafting by day 21; nevertheless, thymic engraftment from L-selectin\textsuperscript{Neg} progenitors was delayed 4 days relative to L-selectin\textsuperscript{Pos} progenitor engraftment, in that L-selectin\textsuperscript{Neg} progenitors did not produce an average of $10^7$ donor-derived thymocytes per engrafted lobe until day 25 (Figure 2A). These results suggest that L-selectin\textsuperscript{Neg} progenitors are more primitive relative to L-selectin\textsuperscript{Pos} progenitors.

Transplantation of $2.5 \times 10^7$ normal thymocytes mediated engraftment of the thymus six days faster than could either L-selectin subset, due to the high dose of cells transplanted and the presence of rapidly expanding progenitors in the thymus.\textsuperscript{13,28} However, both L-selectin subsets were capable of producing $10^6$ donor thymocytes within approximately the same time-frame as could a thymocyte graft (14 days; Figure 2A). Similarly, experiments with grafts enriched for HSC did not produce thymic engraftment until day 21, and required until day 28 to produce $10^7$ donor-derived thymocytes per thymic lobe.\textsuperscript{14} These data indicate that both L-selectin populations
Figure 2: Kinetic analysis of engraftment following IV transplant of L-selectin-positive and -negative bone marrow populations. Panel A: Progenitors were collected from B6-Thy-1.1-Ly-5.1 mice and transplanted IV into sublethally irradiated (6.5 Gy) B6 host mice. Each animal received either $10^3$ L-selectin$^{\text{Pos}}$ progenitors (■), $10^3$ L-selectin$^{\text{Neg}}$ progenitors (○), or $2.5 \times 10^7$ normal thymocytes (Δ). Thymic lobes were collected on the days indicated and assayed by immunofluorescent staining and flow cytometry for donor-derived (Ly-5.1$^{\text{Pos}}$ Thy-1.1$^{\text{Pos}}$) thymocytes. Thymic lobes were considered engrafted if they contained $\geq 10^5$ donor-derived thymocytes. Each symbol represents the mean donor-derived thymocytes per engrafted thymic lobe observed at that time point from 6 to 18 total lobes assayed over three independent sorting and transplant experiments. Error bars
Panel B: Host mice receiving L-selectin$^{\text{Pos}}$ progenitors for the experiment described in panel A were analyzed for the frequency of engrafted thymic lobes relative to the kinetics of thymocyte expansion. The figure shows the percent positive thymic lobes at each time point (right axis, □) and the mean donor-derived thymocytes per engrafted lobe (left axis, ■). Error bars represent SEM. Panels C and D: Bone marrow was collected from the cohort of 62 mice transplanted with L-selectin$^{\text{Pos}}$ progenitors (■) and 48 mice receiving L-selectin$^{\text{Neg}}$ progenitors (○) for the experiment described in A and analyzed by immunofluorescent staining and flow cytometry to identify donor-derived cells expressing either CD19 (B cells) or Ly-6G (granulocytes and macrophages). Each symbol represents the mean donor-derived B cells (Panel C) or Ly-6G cells (Panel D) observed per engrafted femur at the indicated times. Error bars represent SEM. Ly-6G$^{\text{Pos}}$ engraftment following L-selectin$^{\text{Pos}}$ progenitor transplant was too sporadic to extrapolate a trend.

within the Thy-1.1$^{\text{Neg}}$ compartment mediate thymic engraftment with kinetics similar to thymocytes.

After the initial burst of thymic colonization from both L-selectin subsets, distinctly different kinetics emerged. Following day 21, thymic reconstitution mediated by the L-selectin$^{\text{Pos}}$ subset dropped precipitously to less than $10^6$ progeny per lobe on day 25. This span of engraftment reflected the 11 day engraftment cycle observed in normal thymocyte transplants. However, unlike engraftment from thymocytes, thymic colonization following L-selectin$^{\text{Pos}}$ progenitor transplant resurged for a second 11 day cycle, peaking above $10^7$ donor-derived thymocytes per engrafted lobe at day 34 before dissipating between days 40 and 56 post transplant (Figure 2A).

The frequency of engrafted thymic lobes over time reflected the sinusoidal pattern of thymocyte expansion (Figure 2B). However, engraftment frequency (dashed line) was distinctly in advance of the observed expansion kinetics (solid line). The greatest number of engrafted
thymic lobes preceded the greatest thymocyte expansion by a full week. This pattern repeated itself over the second wave of engraftment, with 100% engraftment on day 28, and peak donor thymocyte levels (2.9 x 10^7 ± 1.3 x 10^7) following six days later. By this time, engraftment frequency had decreased to 88%.

L-selectin− progenitors mediated thymocyte expansion that exceeded 10^7 donor-derived cells per engrafted lobe within 25 days post transplant. Unlike expansion following transplant of the L-selectin+ subset, however, this level of engraftment persisted for the eight-week duration of the study (Figure 2A). Likewise, the frequency of engrafted thymic lobes increased steadily through day 28, when an engraftment frequency of 100% was achieved and then maintained for the remaining four weeks of the study. Similar to the L selectin− population, HSC also generate persistent thymic progeny in irradiated hosts but require an additional week to do so. The marked difference in engraftment kinetics between the L-selectin+ and L-selectin− populations confirms that L-selectin expression separates functionally distinct T lineage progenitors within the Thy-1.1− compartment.

Expression of L-selectin coincides with increased bias for the T lineage among Thy-1.1− progenitors

Analysis of B lymphoid and myeloid engraftment in the bone marrow showed distinct differences in lineage potentials of the subsets of cells defined by L-selectin expression. Both subsets contributed to the B lineage during the first two weeks post-transplant (Figure 2C), and the numbers of B cells per femur subsequently decreased in both cases. However, while the L-selectin− cells maintained high numbers of B lineage cells from week 3 onwards, the L-selectin+ cells failed to maintain production of B cells. Further, L-selectin+ cells produced few myeloid progeny in comparison to the L-selectin− subset (Figure 2D). These data demonstrate that L-selectin expression correlates with decreased myeloid and B lineage potential among Thy-1.1− progenitors, a lineage potential that is very similar to the thymic ETP population. Combined with the rapid, cyclic thymic engraftment kinetics discussed above, as well as
phenotypic similarities between the two populations, these data indicate that the $L$-selectin$^{\text{Pos}}$ population includes bone marrow progenitors highly analogous to the thymic ETP population.

**The $L$-selectin$^{\text{Neg}}$ population is highly enriched for B cell precursors**

The diverse engraftment mediated by the $L$-selectin$^{\text{Neg}}$ population suggested the presence of multipotent progenitors. To evaluate the frequency of progenitors for T, B, and myeloid lineages within this population, we conducted a limiting dilution analysis\(^{29}\) (Figure 3).

![Figure 3: Limiting dilution analysis of $L$-selectin$^{\text{Neg}}$ progenitor lineage potential post IV transplant.](image)

$L$-selectin$^{\text{Neg}}$ progenitors were collected and transplanted IV into radiation-conditioned hosts as described for Figure 2 with doses transplanted per mouse ranging from 5 to 160 cells. From three to 13 mice were transplanted at each dose (70 mice total) over four replicate experiments. Engraftment was assessed between 27 and 29 days post transplant by analyzing host tissues for donor-derived cells in specific lineages. Horizontal bars mark the 37% negative engraftment point predicting active progenitor frequencies, which are shown by vertical bars and dose numbers along the x-axis. Correlation coefficients ($r$) are given for each curve extrapolated from the data. Thymic T cells, ($\square$); splenic T cells, ($\blacksquare$); splenic B cells, ($\bigcirc$); marrow B cells, ($\bullet$); marrow myeloid cells, ($\triangle$).
This experiment indicated that 1 in 66 L-selectin\(^{\text{Neg}}\) progenitors were capable of thymic colonization, while 1 in 149 were capable of generating sufficient thymic progeny to result in detectable CD\(^{\text{4Pos}}\) or CD\(^{\text{8Pos}}\) progeny in the spleen within 4 weeks of IV transplant (Figure 3A). B lineage potential was highly enriched within the L-selectin\(^{\text{Neg}}\) population, as 1 in 9 L-selectin\(^{\text{Neg}}\) progenitors produced detectable CD19\(^{\text{Pos}}\) progeny in the spleen, and 1 in 134 mediated B cell colonization of the marrow that persisted through week four (Figure 3B). Surprisingly, at low L-selectin\(^{\text{Neg}}\) cell doses, myeloid engraftment was sparse (Figure 3C). Collectively, these limiting dilution data indicate that L-selectin\(^{\text{Neg}}\) progenitors are strongly biased toward lymphoid commitment, favoring B relative to T lineage potential. We did not attempt limiting dilution analysis of the L-selectin\(^{\text{Pos}}\) subset due to the variation in the frequency of positive thymic lobes with time (Figure 2B).

**L-selectin fractions show distinct cloning efficiencies and in vitro expansion characteristics**

L-selectin\(^{\text{Pos}}\) and L-selectin\(^{\text{Neg}}\) fractions of the Thy-1.1\(^{\text{Neg}}\) population were also tested in methylcellulose cultures supplemented with stem cell factor, IL-7, Flt-3 ligand, IL-3, IL-6, granulocyte colony stimulating factor and erythropoietin. Cloning efficiency kinetics were tabulated over a series of times between five and twelve days in culture. For both populations, mean cloning efficiencies ranged between 10\% and 60\%; however, at each time point the L-selectin\(^{\text{Pos}}\) fraction produced significantly more colonies per cells plated (p < 0.1; Figure 4A). The peak cloning efficiency observed for both subsets was at nine days in culture, with averages of 59\% and 42\% for L-selectin\(^{\text{Pos}}\) and L-selectin\(^{\text{Neg}}\) fractions, respectively.

Colonies were plucked at each of the times indicated in Figure 4B and examined for viable cells by propidium iodide staining and flow cytometry. Little difference in progenitor expansion potentials was seen between the two subsets at early time points; however, after 6 days in culture, the L-selectin\(^{\text{Neg}}\) fraction produced on average from 2- to 100- fold more viable cells per colony for the remainder of the assay (Figure 4B), in spite of producing significantly fewer total colonies (Figure 4A). Cytokines were only added at the inception of each culture and
Figure 4: In vitro and rescue potentials of L-selectin subsets. Panel A: L-selectin^Neg (○) and L-selectin^Pos (■) fractions were collected and cultured in methylcellulose as described in methods. Colonies visible with the aid of a dissecting microscope were counted at the indicated days of culture and plotted as a percent of total cells plated. Each symbol represent the average of four to eight methylcellulose plates counted over three replicate experiments. Error bars represent SEM. Panel B: Colonies formed by L-selectin- negative (○) and L-selectin^Pos (■) fractions were collected, stained with propidium iodide, and analyzed by flow cytometry. Each
symbol represents the average number of viable cells (PI\textsuperscript{Neg}) per colony analyzed at the times indicated. Eight to 24 colonies were analyzed for each data point. Data was pooled from three replicate experiments and error bars represent SEM. **Panel C:** Viable colonies collected for analysis in panel B were evaluated for Ly-6G and CD19 expression. Bars represent the number of colonies containing ≥30 PI\textsuperscript{Neg} cells that expressed either or both (multilineage) of these markers. A total of 144 colonies from L-selectin\textsuperscript{Pos} progenitors and 128 from L-selectin\textsuperscript{Neg} progenitors were analyzed for panels B and C. **Panel D:** Lethally irradiated (13 Gy) mice were transplanted IV with 10\textsuperscript{3} Thy-1.1\textsuperscript{Neg} c-kit\textsuperscript{Hi} L-selectin\textsuperscript{Neg} (Δ) or Thy-1.1\textsuperscript{Lo} c-kit\textsuperscript{Hi} L-selectin\textsuperscript{Pos} (●) progenitors, or 10\textsuperscript{3} Thy-1.1\textsuperscript{Lo} c-kit\textsuperscript{Hi} HSPC (❖), or were left untransplanted (▪).

were not replenished during the course of these assays, which may explain the reduced viability after day nine. Analysis of CD19 or Ly-6G expression revealed that both L-selectin subsets generated B cells and granulocytes at relatively equal frequencies, but only the L-selectin\textsuperscript{Neg} fraction produced multilineage colonies with combined CD19\textsuperscript{Pos} and Ly-6G\textsuperscript{Pos} cells.

Both L-selectin subsets predominantly generated Ly-6G\textsuperscript{Pos} cells in culture and relatively few CD19\textsuperscript{Pos} cells (Figure 4C), although the frequency of CD19\textsuperscript{Pos} colonies produced by the L-selectin\textsuperscript{Neg} population approximates the frequency of B lineage progenitors detected in the in vivo limiting dilution analysis (Figure 3B). These results agree with previous data indicating that the unseparated Thy-1.1\textsuperscript{Neg} c-kit\textsuperscript{Hi} population primarily generates myeloid progeny in culture,\textsuperscript{30} and further supports research suggesting that in vitro lineage potential does not necessarily reflect in vivo transplant performance due to the higher sensitivity of in vitro assays for detecting progenitors with low proliferation potentials.\textsuperscript{31,32}
L-selectin$^\text{Neg}$ progenitors cannot rescue lethally irradiated mice

Like L-selectin$^\text{Neg}$ progenitors, HSC are also Lin$^\text{Neg}$, Sca-1$^\text{Pos}$, and c-kit$^\text{Hi}$. In mice expressing the Thy-1.1 allele of CD90, HSC are confined to the Thy-1.1$^\text{Lo}$ portion of this compartment.$^{22}$ Interestingly, nearly one third of this fraction is also L-selectin$^\text{Neg}$ (Figure 1B). Previous studies have suggested that HSC do not express L-selectin.$^{25,33}$ The high frequency of multilineage engraftment following IV transplantation of the Thy-1.1$^\text{Neg}$ L-selectin$^\text{Neg}$ fraction in the experiments described above, as well as the similarities in phenotype between this population and the stem cell compartment, suggested the possibility that some HSC were also present in the L-selectin$^\text{Neg}$ fraction. In order to rule out this possibility, we transplanted lethally irradiated mice with $10^3$ cells each of Lin$^\text{Neg}$ Sca-1$^\text{Pos}$ c-kit$^\text{Hi}$ Thy-1.1$^\text{Lo}$ HSC, Lin$^\text{Neg}$ Sca-1$^\text{Pos}$ c-kit$^\text{Hi}$ Thy-1.1$^\text{Lo}$ L-selectin$^\text{Pos}$ progenitors, or Lin$^\text{Neg}$ Sca-1$^\text{Pos}$ c-kit$^\text{Hi}$ Thy-1.1$^\text{Neg}$ L-selectin$^\text{Neg}$ progenitors (Figure 4D). All mice were maintained in parallel conditions, and their health was monitored daily. By day 10, all non-transplanted mice had developed white foot pads suggestive of anemia, and by day 14, all members of this cohort had died. Transplants of Thy-1.1$^\text{Neg}$ L-selectin$^\text{Neg}$ progenitors extended the lives of recipient mice for several days beyond the non-transplanted controls; however, all mice had lost color in their foot pads and four mice had expired by day 20. The fifth mouse died on day 26. Similarly, mice receiving Thy-1.1$^\text{Lo}$ L-selectin$^\text{Pos}$ progenitors only persisted until day 28. In contrast, mice receiving Thy-1.1$^\text{Lo}$ c-kit$^\text{Hi}$ HSC never developed white foot pads and all survived through day 35 when the experiment was terminated. The length of time that Thy-1.1$^\text{Neg}$ L-selectin$^\text{Neg}$ and Thy-1.1$^\text{Lo}$ L-selectin$^\text{Pos}$ progenitors sustained transplanted mice suggests that these compartments contain limited platelet and erythrocyte potential. These data confirm that HSC in B6 mice do not express L-selectin.

Discussion

Emerging evidence suggests that during commitment to the T or B lymphoid lineages, a variety of intermediate stages of development are subject to extrinsic controls that can switch the
fate of progenitor cells. The strongest evidence for extrinsically-controlled lineage plasticity comes from experiments in which T or B lineage antigen-specific receptor rearrangements initiate but fail to complete as the cell is redirected to an alternative developmental pathway. In light of these observations, it is difficult to establish lineage relationships in experimental settings since cells isolated from discrete stages of early lymphoid development are likely to respond to experimental conditions and commit to differentiation pathways distinct from those that occur in a natural setting. The role of environmental influences in early lymphopoiesis has clouded recent attempts to resolve the early stages of T and B lineage commitment. We have attempted to address this issue by establishing an experimental model to follow T lineage progenitor activity in prospectively isolated cell populations while minimizing artificial environmental influences such as cell culture in hematopoietic cytokines. In developing our assay, we reasoned that T lineage progenitors should rapidly and efficiently home to the thymus following IV transplantation, and proliferate sufficiently to generate a sizable population of thymocytes within a short period of time. We also evaluated reconstitution of bone marrow and spleen to detect myeloid and B lymphocyte progeny of injected cells, and to follow the emergence of mature T lymphocytes after transplantation. Using this model system, we hope to identify the primary differentiation pathway that leads from the bone marrow to the majority of mature peripheral T lymphocytes.

One difficulty encountered in the dissection of the hematopoietic hierarchy is the phenotypic similarities between progenitor populations and HSC. We have approached this problem by relying on our previous observation that the Thy-1.1 molecule is expressed by virtually all HSC in B6-Thy-1.1 mice. Negative selection for Thy-1.1 expression within the Sca-1^Pos Lin^Neg bone marrow compartment eliminates HSC activity and enriches for lymphoid progenitors, in a manner analogous to selection for Flk-2/Flt3 expression among Sca-1^Pos Lin^Neg cells. Within the Thy-1.1^Neg population, three subsets of cells can be resolved based on levels of c-kit expression; all three of these subsets have been shown to include T and B lymphoid progenitors. The most primitive of these, based on biologic activity and gene
expression analysis, is characterized by activation of the recombinase gene loci and is found within the \( \text{c-kit}^{\text{High}} \) fraction of \( \text{Sca-1}^{\text{Pos}} \ \text{Lin}^{\text{Neg}} \) bone marrow. These cells, termed early lymphocyte progenitors (ELP), reconstitute the thymus after IV transplantation and also generate B lymphocytes as well as NK cells. ELP do not express IL7-R\( \alpha \), which along with the \( \text{c-kit}^{\text{Low}} \) phenotype is the characteristic marker of CLP. While capable of producing both T and B lineages in clonal assay models, CLP are deficient in thymic homing potential and thus are relatively inefficient progenitors of the T lineage in IV transplant experiments. Recent experiments have identified a second type of CLP (CLP-2), which is characterized by a lack of c-kit expression and a \( \text{B220}^{\text{Pos}} \ \text{CD19}^{\text{Neg}} \) surface antigen phenotype. These cells show a remarkable ability to accumulate in the thymus after IV transplantation, although their ability to proliferate within the thymus is limited; short-term cell culture indicates that these cells are progeny of CLP. Based on c-kit levels and B220 expression, the cell populations characterized in the studies reported here do not overlap with either type of CLP. Our previous work has indicated persistent thymic reconstitution in the IV transplant model only after transplantation of \( \text{c-kit}^{\text{high}} \) cells. Analysis of early thymic subsets has identified the thymic ETP, which apparently arises independently of CLP and therefore of CLP-2 as well. Attempts to integrate all of these subsets into a unified model of lymphoid development are frustrated by the probability that the developmental fate of cells at this stage of development is not absolutely tied to surface antigen expression, and that environmental influences introduced by culture manipulations and transplantation are likely to influence lineage commitment. Furthermore, it is likely that multiple pathways can lead to the development of T cells in the thymus.

In this study, we have focused on the \( \text{c-kit}^{\text{High}} \) subset of \( \text{Sca-1}^{\text{Pos}} \ \text{Lin}^{\text{Neg}} \) bone marrow, a population that includes the ELP. We have shown that L-selectin expression separates this population into two subsets that are functionally distinct. Although both of these harbor considerable T lymphocyte potential, L-selectin\( ^{\text{Pos}} \) progenitors are strongly biased to the lymphoid lineages following IV transplantation and produce a single wave of B lineage engraftment along with recurrent waves of thymic engraftment which occur in the absence of
appreciable recurrence of B lineage or myeloid engraftment (Figure 2). As a result of these observations, we consider the L-selectin$^{\text{Pos}}$ subset to be strongly biased toward T lineage development and suggest that this subset of cells includes the bone marrow counterpart of the thymic ETP population. L-selectin$^{\text{Pos}}$ progenitors share several characteristics with thymic ETP, including the Lin$^{\text{Neg}}$ Sca-1$^{\text{Pos}}$ c-kit$^{\text{Hi}}$ phenotype and the expression of L-selectin (Figure 1C). Both subsets express IL-7R$\alpha$ receptor at frequencies much lower than is reported for CLP, and the thymic ETP have further been shown to be IL-7 unresponsive in vitro. Additionally, previous studies have shown that cells at the earliest stages of thymic development are negative for Thy-1.1 antigen expression, and that DN thymocytes are predominantly L-selectin$^{\text{Pos}}$.

L-selectin$^{\text{Pos}}$ progenitors and thymic ETP also share striking functional similarities, in that both populations predominantly generate T-lineage progeny post transplant, with fewer B cell and scarce myeloid progeny (Figure 2A, C, D). Allman and colleagues reported that thymic ETP colonize the thymus poorly post IV transplant, although 400 thymic ETP transferred intrathymically generated $10^5$ thymocytes within two weeks. In contrast, $10^3$ L-selectin$^{\text{Pos}}$ progenitors transplanted IV reliably generate more than $10^5$ thymic progeny within 14 days (Figure 2A). The difference in thymic reconstitution following IV transplantation suggests that this ability is rapidly lost after thymic colonization by bone marrow progenitors. The observation that L-selectin$^{\text{Pos}}$ progenitors repopulate the thymus following IV transplant with kinetics similar to those of thymic ETP following intrathymic transplant shows the high degree of T-lineage competency inherent within the L-selectin$^{\text{Pos}}$ population.

The extent to which L-selectin$^{\text{Pos}}$ progenitors colonize the thymus immediately post-transplant is unknown. The kinetic analysis shown in Figure 2A indicates that the thymus contains progenitors that can reconstitute thymocytes slightly faster than can L-selectin$^{\text{Pos}}$ progenitors following IV transplantation. Additionally, the distinct waves of thymic colonization that follow L-selectin$^{\text{Pos}}$ progenitor transplant indicate that at least a portion of the transplanted cells colonize the bone marrow and function to re-seed the thymus. Foss and colleagues observed a similar duration between waves of thymic seeding in irradiated hosts. This
correlation may indicate that L-selectin\textsuperscript{Pos} progenitors are sufficiently lymphoid-biased to respond to the native signals that initiate normal thymic seeding. It is likely that both L-selectin\textsuperscript{Neg} and L-selectin\textsuperscript{Pos} progenitors initially seed the marrow, where they expand and respond to the overwhelming demand for leukocytes created by the radiation conditioning of the host. Following this initial engraftment phase, the L-selectin\textsuperscript{Neg} population continues to seed several leukocyte lineages in a consistent, stem-cell-like manner. However, the L-selectin\textsuperscript{Pos} population is already biased to the T lineage; therefore, after the initial demands of crisis hematopoiesis are satisfied, L-selectin\textsuperscript{Pos} progenitors continue to expand as primitive pro-T cells in the marrow until thymic niches become available for engraftment. Further experiments will be required to test this hypothesis.

In addition to the steady thymic engraftment kinetics generated by L-selectin\textsuperscript{Neg} progenitors, limiting dilution analysis indicate that this population is predominated by B cell potential. One in 9 L-selectin\textsuperscript{Neg} progenitors proved capable of generating more than 10\textsuperscript{5} splenic B cells following IV transplant in these assays (Figure 3B). This compares to one in 66 L-selectin\textsuperscript{Neg} progenitors that were capable of repopulating a thymic lobe with greater than 10\textsuperscript{5} thymocytes (Figure 3A). These data suggest that the L-selectin\textsuperscript{Neg} compartment is highly enriched for relatively primitive lymphoid progenitors, some of which may be committed to the B cell lineage. Single-cell studies will be necessary to establish this conclusively.

Our experiments do not address the role of L-selectin expression in early T-lineage development. Indeed, gene targeting studies have shown that L-selectin is not required for this process;\textsuperscript{41,42} however, L-selectin is necessary for naive T cells to home to and traverse high endothelial venules and enter lymph nodes.\textsuperscript{42} Further, L-selectin allows leukocytes to escape circulation and enter sites of inflammation within tissues.\textsuperscript{41,43,44} Accordingly, it is plausible that L-selectin may play a role in the homing of bone marrow progenitors to the thymus. This concept is supported by the regulation of L-selectin by the transcription factor Ikaros, which is essential for lymphocyte development on many levels.\textsuperscript{45} It has recently been observed that inhibiting Ikaros activity blocks L-selectin expression in hematopoietic cell lines,\textsuperscript{46} resulting in a
reduction of spontaneous migration. Extrapolating this observation suggests that L-selectin expression may aid the migration of primitive prothymocytes from the bone marrow to the thymus. This is supported by the fact that c-kit^{Hi} double negative thymocytes (and therefore the thymic ETP) also express L-selectin (Figure 1C). Any functional role for L-selectin in thymic homing must be redundant, however, since L-selectin itself is not necessary for T cell development. This agrees with data presented by Allman and colleagues, showing that Ikaros-deficient mice still accumulate thymic ETP but lack CLP.13

In addition to describing a bone marrow counterpart to the thymic ETP population, these studies confirm that L-selectin is not expressed by hematopoietic stem and progenitor cells capable of rescuing mice from lethal irradiation (Figure 4D).25,33 Along with expression of Flk-2/Flt337,38 and other markers,47 this property will be useful in refining stem cell isolation within the Lin^{Neg} Sca-1^{Pos} c-kit^{Hi} population. Furthermore, these data suggest that isolating the L-selectin^{Pos} fraction of this primitive bone marrow compartment is an effective way to enrich lymphoid progenitors for specific study in the absence of HSC contamination.

References


L-Selectin Defines a Bone Marrow Analogue to the Thymic Early T-Lineage Progenitor

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