Lin−Sca1+Kit− Bone Marrow Cells Contain Early Lymphoid-Committed Precursors That Are Distinct from Common Lymphoid Progenitors

Ritu Kumar, Valentina Fossati, Mason Israel and Hans-Willem Snoeck

J Immunol 2008; 181:7507-7513; doi: 10.4049/jimmunol.181.11.7507
http://www.jimmunol.org/content/181/11/7507

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

References  This article cites 39 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/181/11/7507.full#ref-list-1

Subscription  Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Lin−Sca1+Kit− Bone Marrow Cells Contain Early Lymphoid-Committed Precursors That Are Distinct from Common Lymphoid Progenitors

Ritu Kumar, Valentina Fossati, Mason Israel, and Hans-Willem Snoeck

The significance of a population in mouse bone marrow of lineage-negative (Lin−), Sca1-positive, c-kit-negative (LSK−) cells, which is reported to be devoid of long-term repopulation capacity or myeloid potential, is unknown. In this study, we show that the LSK− population is composed of several subsets defined by the expression of flt3, CD25, and IL-7Rα. The first subset was CD25− and more than 90% expressed either flt3, IL-7Rα, or both. The CD25− LSK− population had T cell, B cell, and NK cell potential in vivo, and most of this activity was localized in the flt3− subset, irrespective of the expression of IL-7Rα.

Although lymphoid potential of flt3+t Sca1lokitlowIL7R− cells in vivo was 3-fold lower than that of Lin−Sca1highkitlowIL7Rα− common lymphoid progenitors (CLPs), their cloning efficiency in vitro was 10-fold lower than that of CLPs. Furthermore, although the myeloid potential of Flt3L− Sca1lokitlowIL7R− cells was 10-fold lower than that of CLPs in the absence of M-CSF, the relative myeloid potential of both populations was similar in its presence. These observations suggest differential growth factor requirements of both populations. The second subset of LSK− cells was homogeneously CD25+tflt3−IL7Rα+ and could be generated from both CD25+t LSK− cells and from CLPs, but did not engraft in immunodeficient Rag1−/− or Rag1−/−γc−/− hosts. This population, of which the significance is unclear, was increased in Rag1−/− mice and in old mice. Thus, the LSK− population is phenotypically and functionally heterogeneous and contains early lymphoid-committed precursors. Our findings imply that the early stages of lymphoid commitment are more complex than was thus far assumed.

Hematopoietic stem cells (HSCs) can self-renew and generate all lineages of the hematopoietic system (1). In the mouse model, they are enriched in a population that does not express markers of mature hematopoietic cells (i.e., lineage-negative (Lin−) cells), and expresses Sca1 and c-kit (Lin−Sca1+kit− (LSK) cells), but is devoid of flt3 expression (flt3− LSK cells) (2–5). Upon differentiation into multipotential progenitors (MPPs), which do not possess extensive self-renewal capacity, HSCs acquire the expression of flt3 (4, 5). During lymphoid commitment of HSCs, erythroid and megakaryocytic potential progressively decreases, although whether erythroid or megakaryocytic and myeloid potential are sequentially lost during the very early stages of differentiation is still a matter of debate (6–8). Lymphoid commitment of MPPs is accompanied by the sequential up-regulation of the RAG genes in early lymphoid progenitors (ELPs) (9) and of IL7R in common lymphoid progenitors (CLPs) (10). Although CLPs have T cell potential in vitro and in vivo, they may not be obligate precursors for T cells and function predominantly as early B cell progenitors in vivo (11, 12).

An earlier progenitor, probably a HSC/MPP that expresses CCR9, is likely the main T cell precursor that seeds the thymus (13, 14). This contention is further supported by the recent finding that, whereas CLPs are mostly devoid of myeloid potential, early thymic progenitors retain myeloid differentiation capacity (15, 16). In addition, a Lin−IL7Rα+flt3+kitlow population with predominant T cell potential has been identified in the blood (17). Expression of c-kit is maintained throughout the early stages of T and B cell development (18).

In addition to LSK cells, a cell population has been described in the bone marrow with a similar Lin−Sca1+ phenotype but lacking the expression of c-kit (Lin−Sca1−kit− or LSK− cells) (19). LSK− cells could be generated from transplanted LSK cells in vivo and express the pan-hematopoietic marker CD45, suggesting that they are hematopoietic cells. Their function was unknown, however, as they did not possess long-term repopulation capacity and could not be grown in vitro. LSK− cells were furthermore present in Rag1−/− mice, indicating that they are not a mature lymphocyte subpopulation that requires gene rearrangement of Ag receptors. Quite intriguingly, LSK− cells are rare in fetal liver and accumulate with age in the bone marrow (19).

In this study, we show that LSK− cells contain early lymphoid-committed precursors with both T and B cell potential that are functionally and phenotypically distinct from CLPs. In addition, a subpopulation of LSK− cells expresses high levels of CD25, expands with age, and has no lymphoid precursor activity. A similar population can be generated from CD25+t LSK− cells and from CLPs, however, suggesting that, although its function is unknown, the CD25+t LSK− population belongs to the lymphoid lineage.
Materials and Methods

Mice

The 4- to 8-wk-old C57BL/6 (CD45.2) mice and B6.Ly5.2 (B6.Ly5.2tm1Mom) (CD45.1) mice were purchased from the National Cancer Institute animal facility (Frederick, MD). Rag1−/− (B6.129S7-mmtm1Mom) (CD45.2) mice were purchased from The Jackson Laboratory. The 18-mo-old C57BL/6 mice were aged at the Mount Sinai animal facility (New York, NY). Animals were kept in a specific pathogen-free facility. Experiments and animal care were performed in accordance with the Mount Sinai Institutional Animal Care and Use Committee.

Ab conjugates

Unlabeled anti-CD2, CD3e, CD4, CD8α, Mac-1, Gr1, B220, and IgM. FITC-conjugated anti-CD45.1, SpectralRed-conjugated anti-B220, and biotinylated anti-Thy-1 were purchased from Southern Biotechnology Associates. FITC-conjugated anti-TER119, CD2, CD3e, CD4, CD8α, B220, Mac1, Gr1, and TCR-β,allophycocyanin-conjugated CD25, PE-conjugated anti-Flt3 and anti-IL7Rα, PE-Cy7-conjugated anti-IL7Rα, and PE-Cy7-streptavidin were from eBioscience. Unlabeled anti-TER119, allophycocyanin-conjugated anti-IgM, c-kit, and CD44, PE-Cy7-conjugated anti-CD19, PE-conjugated anti-CD25, FITC-conjugated anti-CD19, allophycocyanin Cy7-conjugated anti-CD8, biotinylated and PE-conjugated anti-CD4, anti-Scal and PerCP-Cy5.5 streptavidin were from BD Pharmingen. Flt3 ligand (Flt3L) and IL7 were purchased from R&D Systems.

Preparation of hematopoietic cells

Bone marrow cells were prepared by flushing the femur and tibia of mice with cold DMEM (Cellgro; Mediatech) containing 2% FBS and 100 ng/ml penicillin/streptomycin. Thymus and spleen cell suspensions were prepared by mincing the organs through nylon mesh. Mononuclear cells were obtained after gradient centrifugation using lymphocyte separation medium (Cellgro; Mediatech).

Flow cytometry

Low-density bone marrow cells, thymus, or spleen cell suspensions were prepared by mincing the organs through nylon mesh. Mononuclear cells were obtained after gradient centrifugation using lymphocyte separation medium (Cellgro; Mediatech).

Reconstitution of Rag1−/− mice

Sorted CLPs or LSK− cells and subpopulations therefrom of bone marrow of CD45.1+ mice were injected in the tail vein of sublethally irradiated (500 cG) Rag1−/− mice. Cell doses ranged from 500 to 1500 cells per mouse. Donor-derived cells were distinguished by expression of CD45.1.

Quantitative PCR

Absolutely RNA Nanoprep kit (Stratagene) was used according to the manufacturer’s instructions. RNA was treated with Dnase I and reverse transcribed into cDNA using random hexamers with SuperScript first-strand synthesis kit for RT-PCR (Invitrogen), Real-time quantitative PCR was performed on ABI 7900HT thermocycler (Applied Biosystems) with a -10 min step at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. All experiments were done in triplicate with SYBR GreenER quantitative PCR SuperMix (Invitrogen). Primer sequences used were the following: Rag1 (sense) 5’-ACCCCTGATCCATGGTTCG-3’, (antisense) 5’-GCCCTTCAAGGATCTCCACC-3’; Rag2 (sense) 5’-TGAACCCACATACGCCATTCT-3’, (antisense) 5’-TTGCTCTTCTGCAGTACAGCGC-3’; Notch1 (sense) 5’-TAACTGCGGAGGATGGTGAGG-3’, (antisense) 5’-GGCCGAAAAAGTGGGAAGGTG-3’; and GAPDH (sense) 5’TGCAGTCCTTCCACCTGCAAAC-3’, (antisense) 5’-GGATGGTGTTGAAACCAAGGAG-3’. Relative quantification was obtained in relation to a standard curve. The standard curve was created using total RNA from sorted double negative (DN) thymic progenitors through a 10-fold dilution series of cDNA standards ranging from 100 ng/μl to 0.1 ng/μl. Quantified values for each gene of interest were normalized against the input determined by the housekeeping gene GAPDH. Combined data from three independent triplicate experiments were normalized to the data obtained for CLPs.

D4-10J1 gene rearrangements

Genomic DNA from 10,000 sorted LSK−/CD25+ cells and CLPs cells was extracted using QIAamp DNA micro kit (Qiagen), following the manufacturer’s instructions. D4-10J1 rearrangements were analyzed by nested PCR following the protocol previously described by Borghesi et al. (20).

OP9 cultures

OP9-Mig R1 (OP9) cells and OP9-DL1 were provided by J. C. Zúñiga-Pflücker (University of Toronto, Toronto, Ontario, Canada). The 1000 sorted cells were seeded in 6-well culture plates containing a monolayer of OP9 cells. Culture medium was α-MEM (Cellgro; Mediatech) containing 20% FBS (HyClone Laboratories), 100 ng/ml penicillin/streptomycin, and 5 ng/ml recombinant mouse IL-7 and Flt3L and, in some experiments, M-CSF (10 ng/ml). Cultures were harvested after 7–14 days for analysis by flow cytometry. Hematopoietic cells were identified as cells with a low forward and side light scatter that did not express GFP.

Statistical analysis

Student’s t test for unpaired samples was used, unless otherwise indicated. A value of p < 0.05 was considered indicative of statistical significance.

Results

Lymphoid potential of LSK− cells

A population of lin−c-kit+ cells isolated from the bone marrow by elutriation has been suggested to contain precursors of long-term repopulating HSC by Ortiz et al. (21). The LSK− cells described by Randall and Weissman (19) are likely different, however, as the cells described by the Ortiz study expressed variable levels of Sca1, whereas LSK− cells express high levels of Sca1 (Fig. 1a). Furthermore, in our hands, LSK− cells had no long-term competitive repopulation capacity, even when recipient mice were analyzed 12 mo after transplantation (data not shown), confirming previously published data (19).

LSK− cells do not grow in colony assays, suggesting that they have limited or no myeloid or erythroid potential. Their lymphoid potential was, however, never tested. To begin to examine the lymphoid potential of LSK− cells, we plated these cells in the presence of OP9 stromal cells, IL-7 and Flt3L, conditions that support B cell development in vitro (22). After 7–14 days of culture, B220+CD19+ (compatible with prepro-B cells) and B220−CD19+ (compatible with pre- and pre-B cells) (23) populations were detected (Fig. 1b, top panels). Next, we tested T cell potential in vitro. After plating in the presence of OP9-DL1 cells, which support the development of T lineage cells (24), LSK− cells generated Thy-1+ cells (Fig. 1b, bottom panels), some of which expressed CD8 (24% after 2 wk, data not shown). The earliest T cell precursors do not express CD3, CD4, and CD8 and are DN. The DN population can be further divided into four populations based on the expression of CD25 and CD44. The earliest T cell precursors are CD25−CD44−, and are termed DN1 cells. These cells proceed to express CD25 (CD25−CD44−) or DN2 cells, then lose the expression of CD44 (CD25−CD44−) cells or DN3 cells) and finally become CD25+CD44− cells (DN4 cells), which begin to express both CD4 and CD8 (double positive cells) (25). LSK− cells plated on OP9-DL1 cells and analyzed after 10 days for the expression of CD25 and CD44 faithfully traversed these consecutive early stages of T cell development (Fig. 1b, bottom panels). Thus, the LSK− population contains cells with T and B cell potential in vitro.

To evaluate the lymphoid potential of LSK− cells in vivo, we injected 1000 CD45.1+ LSK− cells from CD45.1+ congenic C57BL/6 mice into sublethally (500 cG) irradiated Rag1−/− mice (CD45.2−) and backcrossed for a minimum of 15 generations onto C57BL/6. Donor-derived cells were detected in the spleen and...
thymus after 4 wk. No myeloid cells were observed, and the majority of cells in the spleen were either T or B cells, whereas a small fraction of NK cells was also detected (Fig. 1c). Thus, LSK/H11002 cells have T cell, B cell, and NK cell potential in vivo.

Subpopulations of LSK cells defined by IL7R, flt3, and CD25

As LSK cells contain ELP activity but appear devoid of myeloid potential, they are functionally similar to CLPs. CLPs are defined by the lin-Scalow-kitlowIL7R+/flt3+ phenotype (10) and express flt3 (26). LSK cells are phenotypically distinct from CLPs. Although both populations occur with similar frequency in the bone marrow (Fig. 2a) and CLPs are, like LSK cells, by definition negative for lineage markers, but express c-kit and have a lower expression of Scal than LSK cells (Fig. 2a). Furthermore, LSK cells contain cells that are smaller than CLPs, suggesting that LSK cells may be a heterogeneous population (Fig. 2a).

Given the lymphoid potential of LSK cells and the fact that CLPs express flt3 and IL7Rα (10, 26), we analyzed the expression of these cytokine receptors in LSK cells. The 60–80% (n = 8 experiments) of LSK cells expressed IL7Rα (Fig. 2b, top). 40 to 50% of LSK cells also expressed flt3 (Fig. 2b, bottom). In addition, we observed that 50–90% (n = 7 experiments) of LSK cells expressed high amounts of CD25, the α-chain of the IL2R (Fig. 2c, top panel). As LSK cells expressed more CD25 than expressed in any other cell type in the bone marrow (shaded histogram, Fig. 2c, top panel), we call these cells CD25+LSK cells. CD25+LSK cells expressed much more Scal than CD25-LSK cells, and were smaller as judged by their forward scatter profile (Fig. 2d). CD25+LSK cells are not regulatory T cells, however, as they expressed neither CD4 nor Foxp3 (data not shown). Combined analysis of the expression of CD25, flt3, and IL7Rα on LSK cells showed that >85% of CD25+LSK cells were either

---

**FIGURE 1.** Lymphoid potential of LSK cells. a, Sort windows used for the isolation of LSK cells from bone marrow. b, Generation of B lineage cells (top panels) and T lineage cells (bottom panels) 10 days after plating of 1000 LSK cells in the presence of OP9 cells, IL-7, and flt3 (top) or of OP9-DL1 cells (bottom). c, Repopulation of sublethally irradiated Rag1−/− mice 4 wk after transfer of 1000 LSK cells. Representative of eight experiments.

**FIGURE 2.** Subpopulations of LSK cells. a, Comparison of the phenotype of LSK cells and CLPs. Comparison of forward and side scatter of LSK cells and CLPs are shown (bottom middle panel). b, Expression of IL-7Rα and flt3 on LSK cells (open histogram) and on total bone marrow cells (gray shaded histogram) (see Figs. 1a and 2a for representative analysis gates). Representative of eight experiments. c, Expression of CD25 on LSK cells (top panel), and expression of IL-7Rα and flt3 on CD25- and CD25+LSK cells (bottom panels). The shaded histogram (top) is the expression of CD25 on the total bone marrow population. Representative of seven experiments. d, Scal (top) and FSC profile (bottom) of CD25+ and CD25- LSK cells. Representative of seven experiments.
flt3+IL7Ra+ or flt3-IL7Ra-, whereas CD25+LSK− cells were homogeneously flt3+IL7Ra+ (Fig. 2c, bottom panels).

To investigate how expression of IL7Ra, flt3, and CD25 affects the lymphoid potential of LSK− cells in vivo, we injected 1000 IL-7Ra+, IL-7Ra−, flt3+, flt3−, CD25+, and CD25− LSK− cells from CD45.1+ congenic C57BL/6 mice into sublethally (500 cG) irradiated Rag1−/− mice. Both IL-7Ra+ and IL-7Ra− populations generated donor-derived cells after adoptive transfer into Rag1−/− hosts (Fig. 3a). The percentage of donor-derived CD45.1+ cells tended to be higher after adoptive transfer of IL-7Ra− LSK− cells than after adoptive transfer of IL-7Ra+ LSK− cells, although the difference did not reach statistical significance (Fig. 3a). Flt3+ LSK− cells generated ~30-fold more donor cells than flt3− cells (Fig. 3a). CD25− LSK− cells consistently yielded donor-derived cells. In all populations tested, more than 90% of the cells generated were B and T cells, whereas myeloid cells (Mac1+Gr1+) were never observed (data not shown). In contrast, CD25+LSK− cells did not engraft after 3 wk (Fig. 3a). However, when we injected 106 CD25+LSK− cells, a donor-derived CD25+CD19− population was detectable in the bone marrow up to 1 wk after transfer (Fig. 3b). CD25+LSK− cells were not regulatory T cells (data not shown). These data suggest complex density-dependent interactions among the plated cells. Although the curves indicate a much lower proliferative capacity in vivo is significantly lower than that of CLPs.

Next, we compared the proliferative capacity of flt3−LSK− cells with CLPs in vitro by determining the cloning efficiency of these populations in limiting dilution cultures supported by OP9 cells. Surprisingly and in contrast to previously reported data (10), both for CLPs and flt3−LSK− cells, the best fitting curve when the logarithm of the fraction of negative wells was plotted against plated cell number was bicubic (R² = 0.985 for CLPs and R² = 0.903 for flt3−LSK− cells) and not linear (R² = 0.761 for CLPs and R² = 0.543 for flt3−LSK− cells) (Fig. 4f). Similar observations were made in cultures supported by OP9-DL1 cells (data not shown). These data suggest complex density-dependent interactions among the plated cells. Although the curves indicate a much lower proliferative capacity of flt3−LSK− cells than that of CLPs (Fig. 4f), this precludes estimation of the cloning efficiency of these populations using this method, which is based on the premise of single hit kinetics (28, 29). Therefore, we performed single cell sorting experiments. The cloning efficiency of flt3−IL7Ra−LSK− was at least 10-fold lower than the efficiency of CLPs, both in cultures supported by OP9 and by OP9-DL1 cells, whereas in a
capacity of flt3
ments; that in CLPs (CLPs and of flt3 potential (27). Therefore, we compared the myeloid potential of Notch1 mRNA.
kit express less c-, and in the
Gated on cells with low to intermediate FSC and low SSC (see Fig. 1 a) conclude that the myeloid potential of flt3
vide an obvious explanation for the lower proliferative capacity of
receptor is essential for T cell development (24). By quantitative
LSK cells, it was difficult to impossible to establish whether any
cells than in CLPs (Fig. 4 b). However in the presence of M-CSF,
for CLPs normalized to 1 in each experiment). Thus, proliferative
cells to donor-derived cells in the spleen after transfer of 1500 CLPs, IL-7R
LSK cells contained physiologically relevant early lymphoid pre-
If LSK cells contain physiologically relevant early lymphoid pre-
cells but not of CD252 LSK cells from Rag1−/− mice compared with wt mice (data not shown). As bone marrow cellularity was similar in Rag1−/− and wt mice (data not shown), the increase in frequency represents an increase in absolute number. Next, we examined LSK− cells and CLPs in older mice, as it has been shown that the frequency of LSK− cells increases with age (19), whereas the frequency and function of CLPs declines (30, 31). Confirming these previously published findings, we observed that the frequency of LSK− cells was ~5-fold higher in 18-mo-old than in 2-mo-old mice, whereas the frequency of CLPs was 2- to 3-fold lower in old compared with young mice (Fig. 5a). A similar decline in flt3− cells was observed among LSK− cells (Fig. 5b), indicative of an age-related decline in MPPs, as has been

total of more than 400 wells, no colonies derived from flt3− IL7Ra− LSK− cells were ever observed (Fig. 4g). There were no obvious differences in the size of the colonies generated from CLPs or from flt3− IL7Ra− CD252 LSK− cells (data not shown). Although the lower expression of c-kit, and in the flt3− IL7Ra− LSK− fraction the expression of IL-7Ra, might provide an obvious explanation for the lower proliferative capacity of flt3− LSK− cells, we also measured expression of Notch1, as this receptor is essential for T cell development (24). By quantitative PCR, Notch1 expression in flt3− LSK− cells was 42 ± 0.15% of that in CLPs (p = 0.059, n = 3 independent triplicate experiments; p < 0.05 in each individual experiment). Thus, proliferative capacity of flt3− LSK− is lower in vivo and particularly in vitro compared with CLPs. Furthermore, flt3− LSK− cells do not only express less c-kit than CLPs, they also express lower levels of Notch1 mRNA.

The CLP population has been shown to retain some myeloid potential (27). Therefore, we compared the myeloid potential of CLPs and of flt3− LSK− cells by plating the cells on OP9 cells in the presence of IL-7, Flt3L with or without M-CSF. Myeloid potential was expressed as the ratio of B cells to myeloid cells (de-

**FIGURE 4.** Functional distinction between CLPs and flt3− LSK− cells. a, DpJ4 gene rearrangements in purified CLPs and flt3− LSK− cells analyzed by nested PCR (20). b, Quantitative PCR of the expression of Rag1 and Rag2 in CLPs and in flt3− LSK− cells (n = 3 triplicate paired experiments, data for CLPs normalized to 1 in each experiment). c, Percentage of donor-derived cells in the spleen after transfer of 1500 CLPs, IL-7Ra−, or IL-7Ra− flt3− LSK− cells into sublethally irradiated Rag1−/− mice (n = 4). *p < 0.01, by Wilcoxon signed rank test. d, Contribution of T cells, B cells, and NK cells to donor-derived cells in the spleen after transfer of 1500 CLPs, IL-7Ra−, or IL-7Ra− flt3− CD252 LSK− cells into sublethally irradiated Rag1−/− mice. e, Representative example of repopulation of the spleen 3 wk after cotransplantation of 1500 CD45.1 CD45.2 flt3− LSK− cells and CD45.1+ CLPs. Gated on cells with low to intermediate FSC and low SSC (see Fig. 1 a). f, Best fitting curve in limiting dilution experiments of CLPs and CD252 flt3− LSK− cells in the presence of OP9 cells, IL-7, and Flt3L. g, Cloning efficiency of CLPs, IL-7Ra−, or IL-7Ra− flt3− LSK− cells in single cell sorting and culture experiments in the presence OP9 cells, IL-7, and Flt3L and of OP9-DL1 cells. *p < 0.0001 (n = 3 independent experiments). h, Ratio of B cells (CD19+) to myeloid cells (Mac1+ Gr1+) in cultures of CLPs or of flt3− LSK− cells in cultures supported by OP9 cells in the presence or absence of M-CSF.

**LSK− cells are increased in lymphopenic and in older mice**

If LSK− cells contain physiologically relevant early lymphoid precursors, their number may increase in conditions of lymphopenia. In Rag1−/− mice, the frequency of CLPs and of CD252− LSK− cells but not of CD252 LSK− cells was 2- to 3-fold higher than in wild-type (wt) mice (Fig. 5a). The distribution of flt3 and IL-7Ra was similar on CD252− LSK− cells from Rag1−/− mice compared with wt mice (data not shown). As bone marrow cellularity was similar in Rag1−/− and wt mice (data not shown), the increase in frequency represents an increase in absolute number. Next, we examined LSK− cells and CLPs in older mice, as it has been shown that the frequency of LSK− cells increases with age (19), whereas the frequency and function of CLPs declines (30, 31). Confirming these previously published findings, we observed that the frequency of LSK− cells was ~5-fold higher in 18-mo-old than in 2-mo-old mice, whereas the frequency of CLPs was 2- to 3-fold lower in old compared with young mice (Fig. 5a). However, similar to the situation in Rag1−/− mice, the increase in LSK− cells was entirely due to an increase in the CD252− fraction. Although the expression of IL-7Ra was similar in LSK− cells from old and young mice (data not shown), the expression of flt3 on LSK− cells was lower in old than in young mice (Fig. 5b). A similar decline in flt3− cells was observed among LSK− cells (Fig. 5b), indicative of an age-related decline in MPPs, as has been
demonstrated previously (30, 32). Consistent with the fact that most lymphoid potential of LSK− cells resided in the flt3− fraction, LSK− cells from old mice generated 7-fold fewer donor-derived cells in the spleen after transfer into Rag1−/− mice (n = 3), *p < 0.001.

We conclude that CD25+ LSK− cells expand in both lymphopenic and old mice. In contrast, the fact that in lymphopenic mice CLPs are increased while CD25− LSK− cells remain unchanged suggests differential regulation of these populations. Finally, aging is associated with a decline in CLPs, MPPs, and flt3+ LSK− cells.

Discussion

We have shown in this study that the LSK− population, previously dubbed a “mystery” population (19), contains a novel subset of early lymphoid precursors with T cell, B cell, and NK cell potential. In addition, we have identified a subset of LSK− cells with a very high expression of CD25 and Sca1 that has, using currently available assays, no detectable potential in vivo. The significance of this CD25+ LSK− population therefore remains a mystery.

Our findings raise three questions: what is the role of LSK− cells in vivo, what is origin of LSK− cells and what are the lineage relations between the various subpopulations of LSK− cells? Most, if not all lymphoid potential of the LSK− population is located in the flt3+ LSK− fraction, which is uniformly CD25+. As this population is ~4-fold less frequent and one-third as potent as CLPs, it is likely that their relative importance as lymphoid precursors is minor compared with CLPs. However, lymphoid potential is measured as the capacity to engraft in immunodeficient, sublethally irradiated hosts. These conditions are not necessarily reflective of steady-state, but represent a situation of maximally active feedback mechanisms to compensate for lymphopenia (33).

Although the lymphoid potential of flt3+ LSK− cells is 3- to 4-fold lower than that of CLPs in vivo, it is at least an order of magnitude lower in vitro, and undetectable for the IL7Rα− subfraction, suggesting differential growth factor requirements of both populations. The lower expression of c-kit, Notch1 and IL7Rα may all contribute to the lower proliferative potential of flt3+ LSK− cells in vitro. Differential growth factor requirements of flt3+ LSK− cells and CLPs are also suggested by the fact that the myeloid potential of both populations is vastly different in the absence, but not in the presence of M-CSF. Given the differential response of CLPs and flt3+ LSK− cells to in vitro vs in vivo microenvironments, it is not excluded that these populations will behave differently in steady-state as opposed to lymphopenic conditions. The fact that in Rag1−/− mice CLPs, but not CD25− LSK− cells, are increased compared with wt mice argues in favor of this idea. However, only experiments where specific lineage tracing of the progeny of CLPs vs LSK− cells can be performed will clarify the relative roles of CLPs and LSK− cells in lymphoid development in steady-state.

The phenotype and function of flt3+ LSK− cells suggests that they are early lymphoid precursors at a differentiation stage similar to that of MPPs (IL-7Rα+) and CLPs (IL-7Rα−) (4, 5, 9, 10, 34). These data are consistent with the observation that the status of Dnase1s rearrangements is similar in CLPs and flt3+ LSK− cells, and that flt3+ LSK− cells, CLPs and MPPs, defined as lin− Sca1+ kit− flt3+ cells, all decline with age (30, 32). Flt3+ LSK− cells are distinct from CLPs, however. First, although CLPs express c-kit (4, 5, 10), flt3+ LSK− cells do not. Second, flt3+ LSK− cells express less Notch1 than CLPs. Third, the proliferative potential of flt3+ LSK− cells is lower than that of CLPs in vivo and much lower than that of CLPs in vitro, suggesting differential growth factor requirements of both populations. Fourth, Rag expression is lower in flt3+ LSK− cells compared with CLPs. Fifth, the myeloid potential of flt3+ LSK− cells is much lower than that of CLPs when the cells are cultured in the presence of OP9 cells in the absence of M-CSF, but not in its presence, again indicating differential cytokine responsiveness of both populations. Finally, although CLPs are increased in Rag1−/− mice compared with wt mice, flt3+ LSK− cells are not, suggesting differential regulation of both populations. Thus, CLPs and flt3+ LSK− cells are functionally distinct lymphoid progenitor populations.

The lineage relation between CLPs and flt3+ LSK− cells is unclear. It is possible that multiple early lymphoid differentiation pathways exist and that CLPs, defined as lin− Sca1+kit−IL7Rα+ cells (10), do not contain all early committed lymphoid precursors activity in the bone marrow as was previously assumed. Alternatively, it is possible that flt3+ LSK− are lymphoid progenitors that for some reason failed to develop further. The lymphoid potential of these cells in vivo, however, argues against this possibility. Our findings suggest that the ELP compartment should probably be viewed more broadly than it is currently being defined.

The lineage relations among subpopulations of LSK− cells are unclear as well. Cooperative signaling through flt3, IL-7Rα, and c-kit is essential for early lymphoid development, but none of these three receptors individually is absolutely required (34–40). Within the CD25+ LSK− fraction, >90% of the cells express either flt3 or IL-7Rα or both. The existence of lymphoid committed precursor populations that do not simultaneously express IL-7Rα, c-kit, and flt3 might be explained by the fact that upon differentiation of HSC into lymphoid-committed progenitors, differentiation markers are acquired in a way that is neither abrupt nor synchronous. This notion is supported by the observation by Igarashi et al. (9) that
within the LSK^{+}flt3^{+}Rag^{GFP^{+}} ELP compartment, there is initial heterogeneity in the expression of Rag1 and TdT in individual cells. Similarly, not all cells defined as lymphoid-pruned MPPs (LSK^{high}flt3^{−}CD34^{−}) uniformly express early lymphoid transcripts (6). The same principle may apply to cytokine receptors. c-kit is not essential for lymphopoiesis, although the requirement for this receptor increases with age (40). Interestingly, in young viable c-kit-deficient (Vickid) mice, CLP numbers are severely reduced, whereas prepro- and pro-B cells are not affected (40). These findings suggested that linear development from HSCs over CLPs to prepro-B cells is not obligatory (40), and that kit-independent pathways may exist. It is therefore possible that flt3^{−}LSK^{−} cells might be one of the alternative intermediaries between HSCs and committed B and T cell progenitors.

The role and significance of CD25^{2−}IL7Rα−/−LSK^{−} cells is unclear. No engrafment was observed beyond the first week after transfer into sublethally irradiated, immunodeficient hosts, and the engrafted cells did not show any sign of differentiation in vivo. It is interesting to note, however, that this population increases with age and in conditions of lymphopenia. In particular the latter finding may suggest a link between this subset and lymphopoiesis. One explanation for their lack of engraftment potential may be that CD25^{2−}LSK^{−} cells are not capable of homing from the bloodstream to the bone marrow. However, the fact that these cells could be detected in the bone marrow up to 1 wk after transfer argues against this idea. An alternative explanation may be that CD25^{2−}LSK^{−} cells are in fact not lymphoid precursors. However, the finding that the majority of CD25^{2−}LSK^{−} cells express IL7Rα, a receptor required for B cell development in adult mice (35–37), in addition to CD25, a lymphoid lineage associated receptor, argues in favor of the lymphoid nature of this cell population. Furthermore, the observation that both CLPs and CD25^{−}LSK^{−} cells can generate a cell type that is phenotypically very similar to CD25^{−}LSK^{−} cells strongly suggest that these cells belong to the lymphoid lineage. It is possible that CD25^{2−}LSK^{−} are in fact a novel type of mature lymphoid cell of which we failed to assay the function.

In conclusion, our observations assign for the first time a function to at least a subfraction of LSK^{−} cells, and suggest that the early stages of lymphoid commitment are more complex than was previously assumed.

Disclosures
The authors have no financial conflict of interest.

References