Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow

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Summary

The existence of a common lymphoid progenitor that can only give rise to T cells, B cells, and natural killer (NK) cells remains controversial and constitutes an important gap in the hematopoietic lineage maps. Here, we report that the Lin$^-$IL-7R$^+$Thy-1$^-$Sca-1^{lo}c-Kit^{lo} population from adult mouse bone marrow possessed a rapid lymphoid-restricted (T, B, and NK) reconstitution capacity in vivo but completely lacked myeloid differentiation potential either in vivo or in vitro. A single Lin$^-$IL-7R$^+$Thy-1$^-$Sca-1^{lo}c-Kit^{lo} cell could generate at least both T and B cells. These data provide direct evidence for the existence of common lymphoid progenitors in sites of early hematopoiesis.

Introduction

Adult bone marrow contains hematopoietic stem cells (HSCs), which can give rise to all lymphocyte populations, as well as other blood cell types (reviewed by Thomas, 1991). In mice, it has been possible to identify and isolate a purified population of HSCs (for reviews, see Visser and Van Bekkum, 1990; Morrison et al., 1995) The Thy-1.1^{lo}lineage(Lin)$^{-/lo}$Sca-1$^+$ population represents only ∼0.05% of mouse bone marrow cells, yet upon transplantation, these cells can fully reconstitute all blood cell elements and radioprotect lethally irradiated congenic hosts (Spangrude et al., 1988). In C57BL/Ka-Thy1.1 mice, this population contains all of the clonogenic multipotent progenitors (Uchida and Weissman, 1992). A single HSC can repopulate all blood cell types (Smith et al., 1991; Osawa et al., 1996). Mouse HSCs have been further subdivided into three populations: long-term HSCs constitute ∼0.005%–0.01% of mouse bone marrow (Harrison and Zhong, 1992; Morrison and Weissman, 1994; Osawa et al., 1996) and can self-renew virtually for life, short-term HSC populations have a limited self-renewal capacity, while multipotent (lympho-myeloid) progenitors do not self-renew (Morrison and Weissman, 1994; Morrison et al., 1997). The three populations of HSCs can be arranged in a lineage, according to a progressive loss of ability to self-renew. The expression markers that are used for purifying these subpopulations within the HSCs include c-Kit (Ikuta et al., 1991; Okada et al., 1991; Ikuta and Weissman, 1992), CD34 (Osawa et al., 1996), incorporation of mitochondrial supravital dye Rhodamine-123 (Mulder and Visser, 1987;

Spangrude and Scollay, 1990; Li and Johnson, 1992), and/or lack or low-level expression of the lineage-associated antigens such as CD4 and Mac-1 (Morrison and Weissman, 1994).

In addition to the loss of self-renewal activity, the coordinated changes of developmental potential of HSCs might also include the irreversible restriction of one or more lineage commitments owing to sequential activation or silencing of various genes (reviewed by Clevers and Grosschedl, 1996; Shivdasani and Orkin, 1996), including genes related to expression of various cytokine receptors (McKinstry, et al., 1997). The identification of oligopotent hematolymphoid progenitors has been difficult, since the evaluation of differentiation potential may be complicated by a failure to detect a differentiation into particular lineages owing to the limitation of the assays available. These bipotent or oligopotent hematolymphoid progenitors may not reach suitable microenvironments in vivo, or may undergo insufficient expansion for detection in vivo, or appear to contain variable differentiation potentials owing to the stochastic nature of lineage commitment, at least in vitro (Nakahata et al., 1982; Suda et al., 1984). Accordingly, the evidence for bipotent or oligopotent progenitors should include demonstration of a strict bi- or oligopotent functional ability that cosegregates with a distinct cell population definable by some markers such as surface phenotypes, and clonal studies showing bi- or oligolineage outcomes from single cells contained in that population.

It is still unclear whether T and B cell development could or must derive from clonogenic lymphoid-restricted stem cells or from common lymphoid progenitors (CLPs), or if they derive from multipotent progenitors. The CD10$^+$CD34$^+$Lin$^-$c-Kit$^-$Thy-1$^-$ population in human bone marrow gives rise to T, B, NK, and lymphoid dendritic (LD) cells, but very few (if any) myeloerythroid cells; they include clonal progenitors for B, NK, and LD cells (Galy et al., 1995), but it is unknown if the single clonogenic cell of this phenotype gives rise to T cells. In the murine thymus, as a population, the most immature Thy-1^{lo}CD4^{lo}CD25$^-$CD44$^+$ or Thy-1^{lo}c-Kit$^+$ thymic precursors can generate all T, B, NK, and LD cells (Wu et al., 1991a; Ardavin et al., 1993; Matsuzaki et al., 1993), and the NK1.1$^+$c-Kit$^+$CD25$^-$CD44$^+$ cells in fetal thymus can generate both T and NK cells (Carlyle et al., 1997). However, clonal analysis of the outcomes of differentiation from these cells have not been reported. Humans with an adenosine deaminase deficiency (Fischer, 1992; Markert, 1994) or mice with ablations or modifications of specific genes such as *Ikaros* and the common cytokine receptor γ chain (γc) lack all three lymphoid classes (Georgopoulos et al., 1994; Cao et al., 1995; DiSanto et al., 1995; Ohbo et al., 1996), but absence of different cells by single gene mutations do not prove existence of a common progenitor (Weissman, 1994). Thus, to date no direct evidence of the existence of cells with a capacity to generate both T and B lymphocytes, but not myeloerythroid cells, has been reported (Shortman and Wu, 1996). We wished to test whether CLPs may exist in sites of early lymphopoiesis at a clonal level.
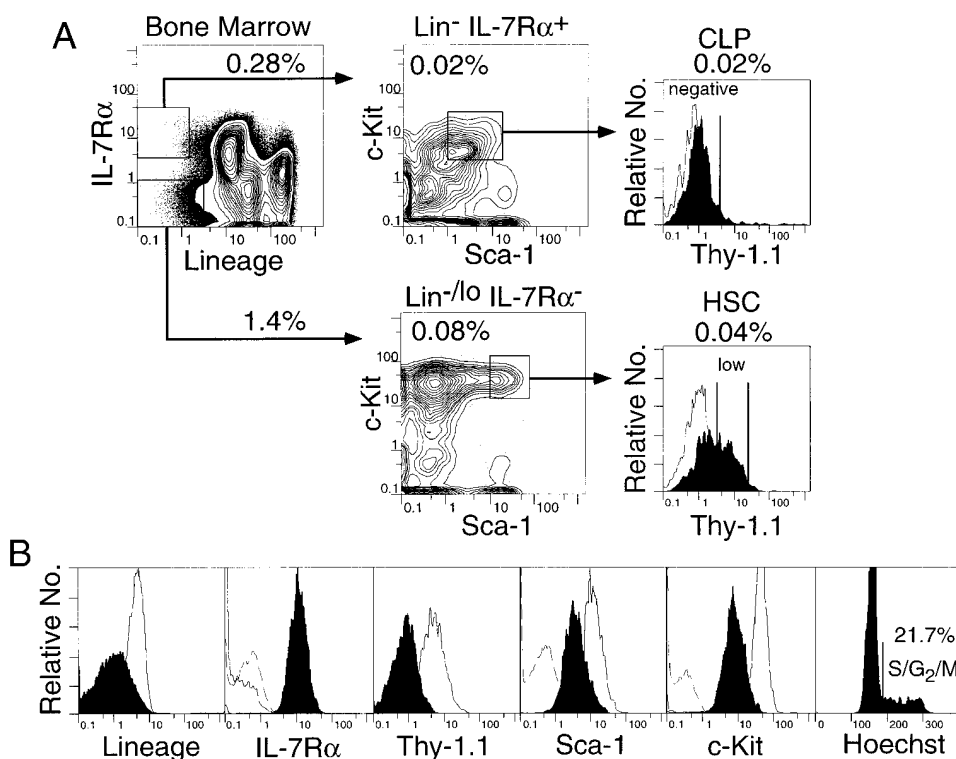
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Figure 1. Identification of IL-7Rα+ Common Lymphoid Progenitors (CLPs) in Mouse Bone Marrow

(A) The cells that stained in the negative to low range for lineage markers (B220, CD4, CD8, CD3, Gr-1, Mac-1, and TER119) were subdivided into IL-7Rα positive and negative fractions (left). Sca-1 and c-Kit profiles of each fraction showed the presence of Lin⁻IL-7R⁺Sca-1ˡᵒc-Kitˡᵒ cells (top center panel) and Lin⁻/ˡᵒIL-7Rα⁻Sca-1ʰⁱc-Kitʰⁱ cells (bottom left panel). More than 90% of the Lin⁻IL-7R⁺Sca-1ˡᵒc-Kitˡᵒ cells were Thy-1.1⁻ (top right panel), and the CLP activity was found in the Thy-1.1⁻ fraction (data not shown). HSCs were purified from the Thy-1.1ˡᵒ fraction of Lin⁻/ˡᵒIL-7Rα⁻Sca-1ʰⁱc-Kitʰⁱ cells (bottom right panel) (Morrison and Weissman, 1994).

(B) The surface phenotype on reanalysis of the sorted candidate CLPs (closed histogram) and HSCs (solid line) and the cell cycle status of the CLP population by Hoechst 33342 staining; dashed lines show negative staining of whole bone marrow cells. The candidate CLPs were Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ, and ~22% of them were in S/G2/M phase of cell cycle.

Interleukin 7 acts as a nonredundant cytokine for both T cell and B cell development (Peschon et al., 1994; von Freeden-Jeffry et al., 1995) by maintaining cell survival for T cell precursors (Akashi et al., 1997; Kondo et al., 1997; Maraskovsky et al., 1997; von Freeden-Jeffry et al., 1997) as well as by supporting proliferation (Namen et al., 1988; Sudo et al., 1989) and rearrangement of immunoglobulin heavy chain (*IgH*) genes for B cell precursors (Corcoran et al., 1996). IL-7 exerts its effect through the interaction of a high affinity receptor complex composed of the IL-7 receptor (IL-7R) α chain (IL-7Rα) and the common cytokine receptor γ chain (Noguchi et al., 1993; Kondo et al., 1994). Because of the pivotal role of the IL-7R-mediated signals in lymphoid development, we searched for CLPs in C57BL/Ka-Thy1.1 mouse bone marrow using IL-7Rα expression as a marker. We demonstrated a newly identified Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ population that possessed rapid and prominent short-term lymphoid-restricted (T, B, and NK cells) reconstitution activity. A clonogenic analysis of the cells revealed that single Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ cells could differentiate into both T and B cells. Thus, this population represents the earliest known lymphoid-committed progenitor in mouse bone marrow.

Results

Identification of the Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ Population

We sorted the bone marrow cells in C57BL/Ka-Thy1.1 mice, using IL-7Rα expression as a marker in addition to conventional HSC markers including Lin, Thy1.1, Sca-1, and c-Kit (Morrison and Weissman, 1994). Figure 1A shows expression profiles of bone marrow cells for these markers. The majority of cells expressing IL-7Rα were B220⁺CD43⁺IgM⁻ pro-B and B220⁺CD43⁻IgM⁻ pre-B cells (Sudo et al., 1993; data not shown). Among the population of cells that did not express lineage markers (Lin⁻), the IL-7Rα⁺ fraction contained a population of Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ cells that made up ~0.02% of total bone marrow cells. All of these IL-7Rα⁺ cells expressed the γc (data not shown), indicating that they expressed functional IL-7R complexes. The Lin⁻/ˡᵒIL-7Rα⁻ fraction contained Thy-1.1ˡᵒSca-1ʰⁱc-Kitʰⁱ cells, which included HSC subsets (Morrison and Weissman, 1994) (Figure 1A). Figure 1B shows a result of reanalysis of the sorted Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ population and the Lin⁻/ˡᵒIL-7Rα⁻Thy-1.1ˡᵒSca-1ʰⁱc-Kitʰⁱ population.

The Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ cells had rearranged neither immunoglobulin heavy chain (*IgH*) nor
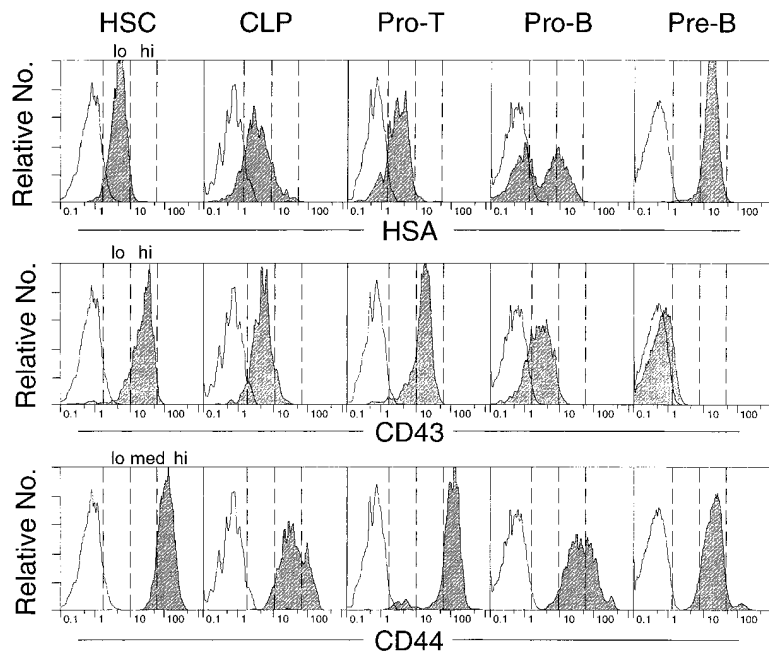
Figure 2. The Expression of HSA, CD43, and CD44 in HSCs, CLPs, and Various Lymphoid Progenitors

The definition of thymic pro-T population used here is CD3-CD4$^-$/^{lo}CD8$^-$/^{lo}CD25$^-$c-Kit$^+$, and that of either bone marrow pro-B or pre-B population is B220$^+$IgM$^-$CD43$^+$ and B220$^+$ IgM$^-$CD43$^-$, respectively (see Experimental Procedures). The gray filled histograms represent the expression profiles of these antigens in each population. Solid lines indicate negative stainings. Since most Lin$^-$IL-7R$^+$ Sca-1^{lo}c-Kit^{lo} cells were Thy-1.1$^-$ (Figure 1), the stainings of HSA, CD43, and CD44 for CLPs were done for Lin$^-$IL-7R$^+$Sca-1^{lo}c-Kit^{lo} cells.

T cell receptor β (*TCR*β) genes determined by PCR analysis (data not shown), and ~22% of the cells were in S/G2/M phase of cell cycle, indicating that considerable fractions are cycling (Figure 1B). Figure 2 shows the expression of heat-stable antigen (HSA; CD24), CD43, and CD44, all have been used previously for identifying either thymic pro-T cells or pro-B cells in bone marrow (Hardy et al., 1991; Wu et al., 1991b). The majority of the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells expressed a low level of CD43 and a medium level of CD44. In contrast, both HSCs and the CD3$^-$c-Kit$^+$CD25$^-$ earliest thymic pro-T precursors (Wu et al., 1991b; Godfrey et al., 1992; Matsuzaki et al., 1993) expressed higher levels of CD43 and CD44. The B220$^+$IgM$^-$CD43$^+$ pro-B cell population in the bone marrow could be divided into HSA$^-$ and HSA^{hi} populations, the former of which corresponded to the most primitive pro-B stage (pre-pro-B; fraction A), according to the classification proposed by Hardy et al (1991). The Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells as well as HSCs and the thymic precursors expressed low levels of HSA. Accordingly, the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells are distinguishable from pro-T and pro-B populations as well as HSCs in terms of expression profiles of these surface markers.

The Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} Cells Possess Rapid and Prominent Lymphoid-Restricted Reconstitution Potential

To evaluate the differentiation capacity of the the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells, we competitively reconstituted lethally irradiated (920 rad) Ly5.1 mice with 2 × 10^3 Ly5.2 congenic Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells and 2 × 10^5 Ly5.1 syngeneic bone marrow cells. Figure 3 shows the serial changes of mean percentages of chimerism in blood nucleated cells after the competitive reconstitution. All through the analysis, we detected exclusively donor-derived T and B cells, but donor-derived Gr-1$^+$ granulocytes or Mac-1$^+$ monocytes never

appeared in the blood. The peak level of donor-type (Ly5.2$^+$) B cells was apparent at 4 weeks (the earliest point of analysis) and of T cells at 4–6 weeks after injection. The percentage of both donor-derived T and B cells gradually decreased, indicating that their self-renewal potential is limited. There was no particular Vβ bias in the donor-derived T cells; they consisted of percentages of Vβ3-, Vβ6-, Vβ8-, and Vβ11-positive cells similar to normal mice (data not shown).

In the competitive reconstitution assay, the coinjected host-type bone marrow cells contained mature lymphocytes as well as a variety of stages of bone marrow
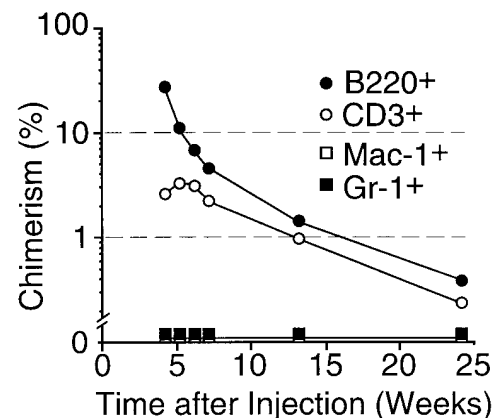

Figure 3. Reconstitution Potential of the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} CLP Population to Lethally Irradiated Congenic Mice

Sequential analysis of blood cell chimerism after competitive reconstitution with 2 × 10^3 CLPs (Ly5.2) and 2 × 10^5 syngeneic (Ly5.1) bone marrow cells. Mean percentages of donor-derived cells positive for B220 (closed circles), CD3 (open circles), Mac-1(open squares), and Gr-1 (closed squares) are shown (n = 4). The B220$^+$ cells in blood were surface IgM$^+$ in all sample tested. Neither Mac-1 nor Gr-1 positive cells were detectable.
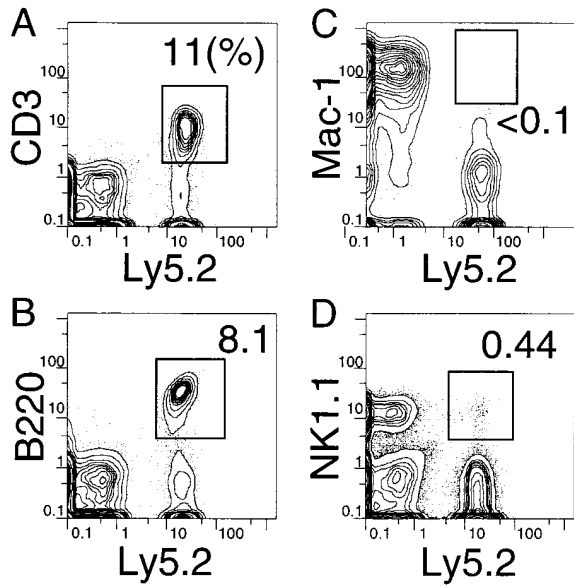
Figure 4. Reconstitution Potential of the Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ CLP Population (Ly5.2) to Sublethally Irradiated *RAG⁻/⁻* (Ly5.1) Mice

(A–C) Phenotypic analysis of nucleated blood cells 4 weeks after intravenous injection of 400 candidate CLPs (Ly5.2) into sublethally irradiated (400 rad) *RAG⁻/⁻* (Ly5.1) mice are shown. The number of donor-type (Ly5.2) CD3⁺ (A) and B220⁺ (B) cells reached 176 cells/μl and 130 cells/μl of blood, respectively, but Mac-1⁺ cells (C) were absent in the blood

(D) CD3⁻NK1.1⁺ cells were also detected in the spleen of the same mouse 8 weeks after injection (the profile of CD3⁻ spleen cells is shown).
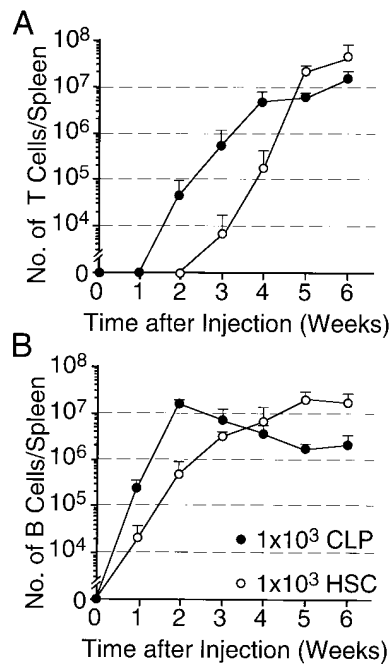

Figure 5. The Sequential Analysis of Numbers of T Cell and B Cell Progeny from CLP and HSC Populations

Absolute numbers of the donor-derived CD3⁺ T cells (A) or B220⁺ B cells (B) in the spleen of sublethally irradiated *RAG-2⁻/⁻* mice injected with either 10³ Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ CLPs (closed circles) or 10³ Lin⁻/ˡᵒIL-7R⁻Sca-1ʰⁱc-Kitʰⁱ HSCs (open circles) were shown. The highest number of CD3⁺ progeny that was generated from 10³ CLPs was ∼1 × 10⁷ at 4–6 weeks, while that of B220⁺ progeny was ∼1.5 × 10⁷ at 2 weeks. Results are shown as mean plus S.D. (error bars) from groups of 3–5 mice at each time point.

precursors including HSCs. We considered the possibility that the Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ cells failed to reach suitable bone marrow microenvironments to read out myeloid outcomes because the stromal niche may be immediately occupied by host-type progenitors or that myeloid differentiation from this population may be perturbed by inflammatory cytokines released from activated lymphocytes as well as damaged stromal cells owing to lethal-dose irradiation (Antin and Ferrara, 1992). To exclude these possibilities, we reconstituted sublethally irradiated syngeneic *RAG-2*-deficient (*RAG⁻/⁻*) mice that lack T and B cells only with the candidate CLP population. We first injected 400 Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ cells (Ly 5.2) to sublethally irradiated (400 rad) Ly5.1 *RAG⁻/⁻* mice. Four weeks after injection, the mice exhibited donor-type reconstitution only for T and B cells, but not myeloid cells (Figure 4). Interestingly, as few as 400 cells could reconstitute the number of blood T and B cells to 5%–20 % of levels in normal mice. We could also detect donor-derived CD3⁻NK1.1⁺ NK cells in the spleen in all 4 mice analyzed 8 weeks after transplantation (Figure 4), indicating that the CLP population can give rise to NK cells as well as to T and B cells.

Next, we evaluated the changes in absolute numbers of T and B progeny in the spleen in the *RAG⁻/⁻* mice that were reconstituted with either 10³ CLP or 10³ HSC (Lin⁻/ˡᵒIL-7R-Thy-1.1ˡᵒSca-1ʰⁱc-Kitʰⁱ) populations. As

shown in Figure 5, 10³ CLPs could generate 0.6∼1.1 × 10⁷ CD3⁺ spleen T cells by 4–6 weeks and 1.4 × 10⁷ B220⁺ spleen B cells by 2 weeks. The peaks of the CD3⁺ T and B220⁺ B cells that were generated from 10³ CLPs appeared ∼7–10 days earlier than similar numbers of T and B cells derived from 10³ HSCs. The changes of blood T and B cell numbers were almost synchronized with those in spleen T and B cells (data not shown). Neither Gr-1⁺ nor Mac-1⁺ myeloid progeny could be detected in either blood, bone marrow, or spleen in these experiments.

Figure 6 shows the sequential changes of CD4 and CD8 profiles of the thymic progeny derived from either 10³ CLPs or 10³ HSCs in the *RAG⁻/⁻* reconstitution assay. In normal thymus, immature CD4⁻CD8⁻ double negative cells rearrange *TCR* genes and proliferate to become CD4⁺CD8⁺ double positive (DP) cells, the DP cells receive a stringent selection determined by MHC-self-peptide and *TCR* interactions, and a minority of DP cells become CD4⁺CD8⁻ or CD4⁻CD8⁺ single positive (SP) cells (Akashi and Weissman, 1996). *RAG⁻/⁻* thymocytes cannot progress to the DP stage owing to the impairment of *TCR* gene rearrangements. As shown in Figure 6, the appearance of donor-type (Ly5.2) DP cells and SP cells was more than a week earlier in the thymus injected with 10³ CLPs (upper panels) than those injected with 10³ HSCs (lower panels). Thus, the CLP population
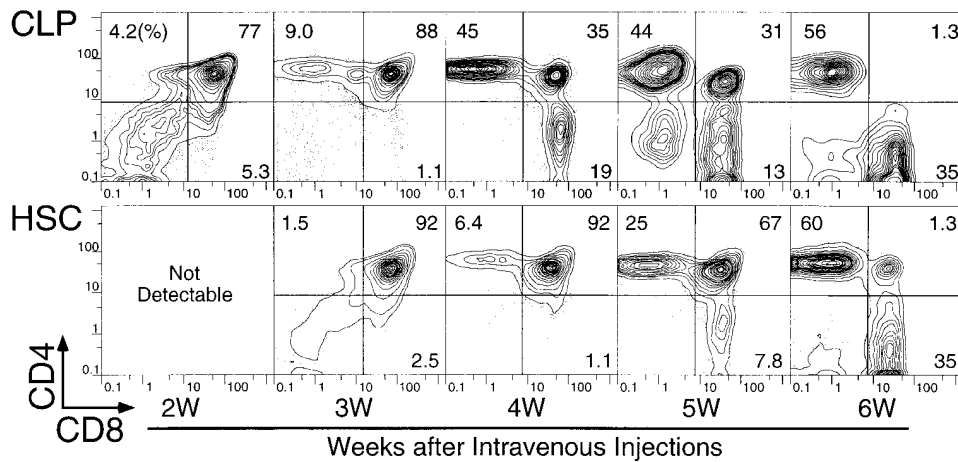
Figure 6. The Sequential Analysis of CD4 and CD8 Profiles of the Thymic Progeny after Reconstitution of RAG$^{-/-}$ (Ly 5.1) Mice with Syngeneic (Ly5.2) 10^3 CLPs or 10^3 HSCs

The appearance of CD4$^+$CD8$^+$ DP cells and CD4$^+$CD8$^-$ or CD4-CD8$^+$ SP cells from 10^3 CLPs preceded those from 10^3 HSCs by more than a week.

possessed a more rapid lymphoid differentiation capacity than the HSC population.

The Differentiation Potential of Lin$^-$IL-7R$^+$ Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} Cells in Limit Dilution Analysis

We evaluated the lymphoid differentiation capacity of the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells at limit dilution, where Poisson statistics would predict that, on average, a single cell can proliferate and differentiate into T or B cells. We competitively reconstituted the lethally irradiated mice with different numbers of the CLP (Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo}) cells with 2 × 10^5 host-type bone marrow. As shown in Figure 7A, approximately 1 in 22 cells can generate B cells after intravenous injection. In this experiment, we could detect donor-type T cell reconstitution in only 1 out of 18 mice that were injected with 35 CLP cells.

To circumvent the problem that T cell differentiation might fail if the cells did not home to the thymic microenvironment, we injected the CLPs directly into the thymus of sublethally irradiated congenic mice, as we did in some of the original assays to define HSCs (Spangrude et al., 1988). The frequency of cells that have the ability to respond to the thymic microenvironment was approximately 1 in 21 cells (Figure 7B). CD4 and CD8 profiles of the donor-derived cells show a normal distribution of CD4 and CD8 SP cells, and DP cells (Figure 7B).

We also checked the B cell differentiation capacity of the CLPs on the Whitlock-Witte-type in vitro culture (Whitlock and Witte, 1982; Muller-Sieburg et al., 1986), using the S17 stromal cell layers (Collins and Dorshkind, 1987). The CLP cells formed B220$^+$IgM$^+$ mature B cell–containing colonies after 14 days (Figure 7C). The frequency of cells that can read out differentiation into B lineage was approximately 1 in 6. Cumano et al. (1992) reported that the bipotent B cell/macrophage progenitor could differentiate into adherent macrophages as well as mature B cells in this culture condition in the presence of macrophage colony-stimulating factor (M-CSF). The

addition of recombinant human M-CSF (25 U/ml) to the medium did not affect the frequency for B cell differentiation, and the cells did not give rise to adherent macrophages (data not shown).

Clonogenic Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} Cells Can Give Rise to B, but Not to Myeloerythroid Colonies in Methylcellulose Culture

We further characterized the differentiation capacity of the CLP population in methylcellulose culture. First, we cultured the cells under the conditions for myeloid colony formation listed in Table 1. We have reported that the frequency of the cells that can respond to the methylcellulose cultures containing steel factor (SLF or c-Kit ligand), IL-3, IL-6, granulocyte/macrophage colony-stimulating factor (GM-CSF) and erythropoietin were ~80% and ~20%–40% in long-term HSC and short-term HSC subsets, respectively (Morrison et al., 1996). However, the CLP (Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo}) cells did not form colonies in various cultures for myeloid colonies (Table 1).

It has been shown that B cell precursors in the bone marrow could form pre-B colonies in methylcellulose containing IL-7 (Suda et al., 1989). However, when we cultured 500 CLPs in the presence of IL-7 alone, only a few colonies were formed on day 7. These colonies were composed of pro-B (B220$^+$CD43$^+$IgM$^-$) and pre-B (B220$^+$CD43$^-$IgM$^-$) cells when analyzed on day 10 (data not shown). We considered the possibility that these earlier progenitors required earlier-acting cytokines. The addition of SLF and/or Flt-3/Flk2 ligand (FL) (Lyman et al., 1993; Hannum et al., 1994) to this culture condition significantly increased the frequency of colony-forming cells: ~20% of the CLP cells formed colonies in the presence of IL-7, SLF, and FL on day 7 (Table 1). These colonies were determined as pro-B/pre-B colonies on day 10 (Figure 7D). When we sorted different numbers of the cells into 96-well plates with methylcellulose containing IL-7, SLF, and FL, the frequency of the cells responding to this culture condition determined on day
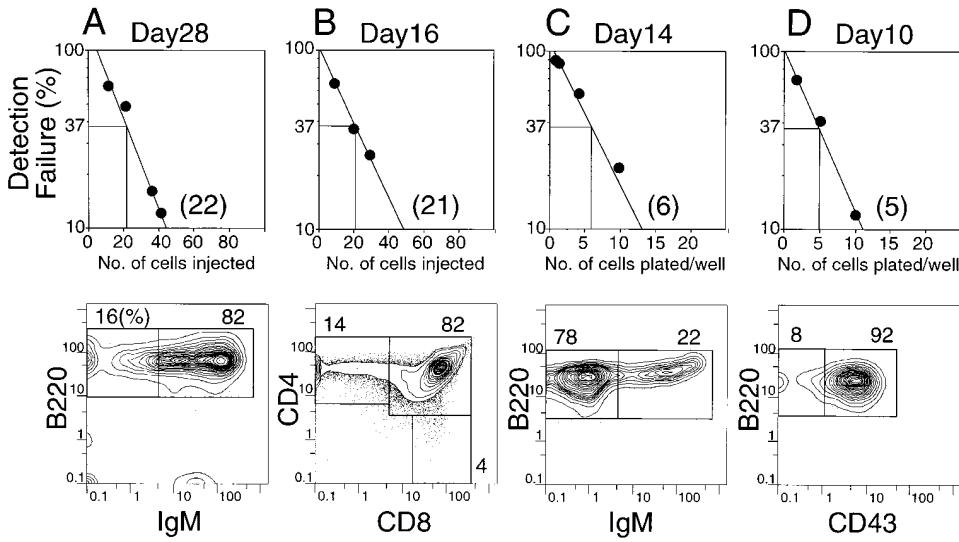
Figure 7. Results of Limit Dilution Analysis of the Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ᴸᵒ-Kitᴸᵒ CLP Population

The differentiation potential of the cells was assessed by intravenous (A) and intrathymic (B) injections after 28 and 16 days, respectively, by culture on S17 stromal layers for 14 days (C), and by culture in methylcellulose containing SLF, IL-7, and FL for 10 days (D). The upper panels show the percentage of failure in detection of progeny from various numbers of cells under each condition. Numbers in parenthesis represent limiting numbers. Lower panels show surface phenotypes of the progeny under each condition. The differentiation outcomes on individual assays were determined as mature B cells in (A), all thymic T cell components in (B), mature B cells in (C), and pro-B/pre-B cells in (D). The surface phenotyping of a colony that grew in methylcellulose (D) was analyzed on day 10.

10 by the limit dilution analysis was also approximately 1 in 5 cells (Figure 7D).

Figure 8 shows the appearance of a day 3 colony derived from a single Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ᴸᵒc-Kitᴸᵒ cell in the limit dilution analysis. The colonies were composed of 20–80 blastic cells. Interestingly, cells randomly changed their positions in methylcellulose along with cell divisions from day 1 to day 2, and the cells constituting the day 3 colony were dispersed, resembling the blast colonies reported by Nakahata and Ogawa (1982) that contained cells capable of self-renewal in vitro and of generating secondary myeloid colonies. We picked cells from the blastic day 3 colonies and replated them into the methylcellulose culture containing SLF, IL-3, IL-6, GM-CSF, M-CSF, and erythropoietin. However, we could not detect secondary myeloid

colonies after replating in these conditions (data not shown).

To confirm that the cells in the day 3 colony possess differentiation potential for mature B cells in vivo, we picked the cells and injected them into lethally irradiated congenic mice with 2×10^5 host-type bone marrow cells. Four weeks after injection, B220⁺IgM⁺ mature B cell progeny could be detected in the spleen from 16 out of 18 injected mice (data not shown). Accordingly, virtually all clonogenic CLP cells in the methylcellulose culture can generate mature B cells, but not myeloid cells.

Single Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ᴸᵒc-Kitᴸᵒ Cells Can Give Rise to Both T and B Cells
For the definition of CLPs, it was important to test

Table 1. Comparison of the Differentiation Potential of CLPs and HSCs in Methylcellulose in the Presence of Cytokines

No. of Day 7 Colonies/500 cells

| | Cytokines Used for Myeloid Colonies | | | | | | Cytokines Used for Lymphoid Colonies | | | | | |
| | SLF + M-CSF | | SLF + IL-3 + IL-6 + GM-CSF + Epo | | SLF + IL-3 + IL-6 + M-CSF + Epo | | IL-7 | | SLF + IL-7 | | SLF + IL-7 + FL | |
Cells	M	B	M	B	M	B	M	B	M	B	M	B
CLP: Lin⁻IL-7R⁺Sca-1ᴸᵒ Thy-1⁻c-Kitᴸᵒ	0	0	0	0	0	0	0	2 ± 1	0	34 ± 5	0	101 ± 8
HSC: Lin⁻ᐟᴸᵒIL-7R⁻Sca-1ʰⁱ Thy-1ᴸᵒc-Kitʰⁱ	1 ± 1	0	240 ± 6	0	154 ± 21	0	0	0	1 ± 1	0	2 ± 1	0

Different media and FCS were used for inducing either myeloid or B lymphoid colonies (see Experimental Procedures). Epo, Erythropoietin; M, myeloid colony included CFU-Mix, CFU-GM, BFU-E, and CFU-Meg (16); B, B lymphoid colonies consisted of pro-B and pre-B cells by phenotype. SLF alone could not stimulate colony-formation in both culture conditions.
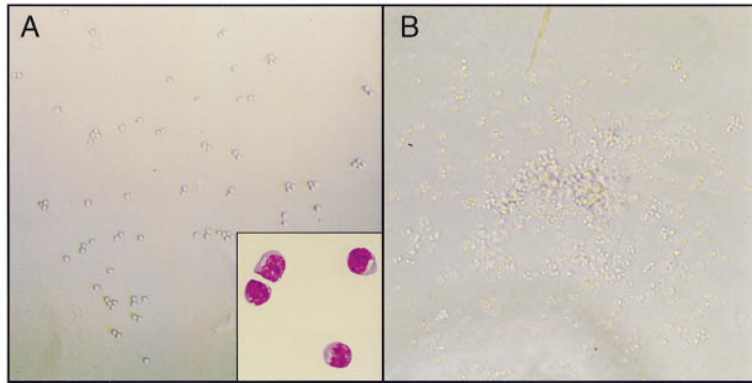Results are shown as mean ± S.D. of three independent experiments.

Figure 8. Colonies Derived from Single Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ Cells in Methylcellulose in the Presence of IL-7, SLF, and FL

(A) The appearance of a day 3 colony that was derived from a single CLP on an inverted microscope and of the cells in the colony on Giemsa staining (right bottom). The colony has a dispersed distribution of blastic cells. (B) The appearance of a single CLP-derived day 7 colony. The colony is composed of B220⁺IgM⁻CD43⁺ pro-B and B220⁺IgM⁻CD43⁻ pre-B cells (see Figure 7D).

whether a single Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ cell could generate both T and B cells. We picked aliquots from the individual single cell-derived day 3 colonies that were grown in methylcellulose containing SLF, FL, and IL-7, and injected these cells intrathymically into sublethally irradiated congenic mice. In all cases, the remaining cells in the single cell-derived colonies continued to grow in methylcellulose and formed pro-B/pre-B cell colonies (Figure 9).

Sixteen days after injection, donor-derived T cells could be detected in 7 out of 20 injected thymuses. Representative data are shown in Figure 8. In 2 out of the 7 thymuses that developed donor-derived T cells, the cells showed normal thymic differentiation into both CD4⁺ and CD8⁺ SP T cells (case 1 in Figure 9, for example). The donor-derived cells contained both CD3⁺ SP cells and CD3ˡᵒ DP cells. Neither myeloid cells nor B cells were detectable in the thymus. In the remaining 5
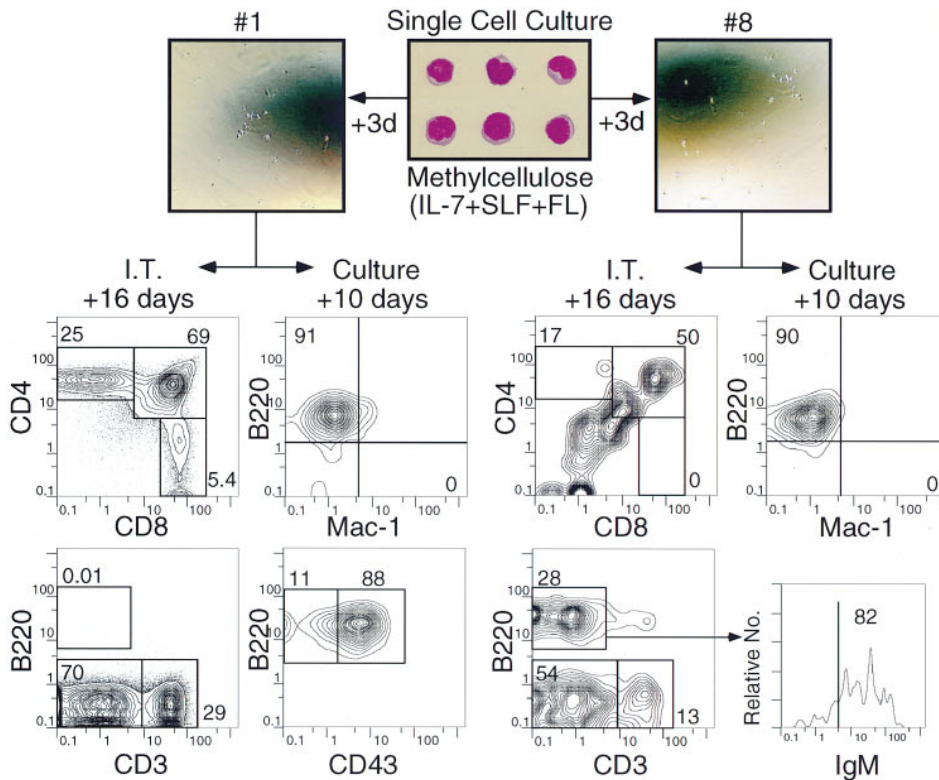

Figure 9. Clonal Analysis of B Cell and T Cell Progenitor Activity of the Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ CLP Population

Morphology of the Lin⁻IL-7R⁺Thy-1.1⁻Sca-1ˡᵒc-Kitˡᵒ cells from C57BL/Ka-Thy1.1 mice (Giemsa stain, top center). Day 3 colonies grew from individual CLP cells (cases 1 and 8) in methylcellulose in the presence of SLF, IL-7, and FL (photographs, top, left and right). The FACS plots show the surface phenotypes of thymic progeny after intrathymic injections (I.T.) of cells picked from single CLP cell-derived colonies and the surface phenotype of cells from the part of the colony that remained and was further cultured on the methylcellulose. Cells that were derived from a single CLP were able to differentiate into mature T cells (case 1) or both mature T and B cells (case 8) in the injected thymuses, and also differentiated into pro-B/pre-B cells in the methylcellulose in both cases.

cases (case 8 in Figure 9, for example), the progeny from a single cell consisted of B220$^+$ B cells as well as CD3$^+$ SP and CD3^{lo} DP cells. The B220$^+$ B cells were surface IgM$^+$ mature B cells. Myeloid cells were not detected. These data collectively indicate that at least some fraction of colony-forming cells in response to SLF, IL-7, and FL retained T-lineage as well as B-lineage differentiation potential after 3 days of the culture.

Discussion

Our data showed that the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo} c-Kit^{lo} population from bone marrow has prominent T and B cell reconstitution potential and contains CLPs that can give rise to at least both T and B cells. This population exhibited no or limited self-renewal activity. The Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells could generate CD3$^-$NK1.1$^+$ NK cells in the spleen after in vivo injection (Figure 4). We have also observed the development of CD3$^-$NK1.1$^+$ NK cells from the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells in vitro after 2 weeks in culture on bone marrow stromal layers upon addition of IL-2 (Aguila et al., personal communication). Thus, the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells likely represent a clonogenic CLP population, though clonal studies for NK cells as well as T and B cells have not been done. Figure 10 summarizes our proposal for CLPs in lymphoid development sequences from HSCs.

The Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells completely lack differentiation capacity to myeloid lineages both in vivo and in vitro. First, neither Gr-1$^+$ nor Mac-1$^+$ myeloid cells could be detected from 1 to 24 weeks after reconstitution in the series of in vivo reconstitution analysis, and 2 × 10^3 CLPs could not give rise to either day 8 CFU-S or donor-derived myeloid (Mac-1$^+$ or Gr-1$^+$) cells in bone marrow 8 days after injection (data not shown). Second, we did not detect myeloid cells, including adherent macrophages, during the culture over 21 days on S17 stromal layers in the presence or absence of M-CSF (data not shown), although the HSCs and the "bipotent" B cell/macrophage precursors reportedly give rise to both B and myeloid cells in this condition (Cumano et al., 1992; Morrison et al., 1996). Third, although HSCs formed a variety of myeloid colonies in methylcellulose in the presence of SLF, IL-3, IL-6, GM-CSF, and erythropoietin in our hands (Morrison et al., 1996), the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} cells did not form myeloid colonies in the culture. The cells constituting day 3 colonies grew in the presence of IL-7, SLF and FL did not give rise to secondary myeloid colonies in the presence of myeloid-directed cytokines in a replating experiment, whereas they could give rise to only T and B cell progeny after in vivo transfer. These data indicate that the differentiating potential of the CLP population is strictly limited to T, B, and NK lineages. Accordingly, this population represents the earliest known stage of lymphoid development distinct from HSCs.

The earliest thymic precursor population could generate B, NK, and LD cells (Wu et al., 1991a; Ardavin et al., 1993; Matsuzaki et al., 1993). The Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} CLP population likely gives rise to LD cells as well as T, B, and NK cells, because ~5% of
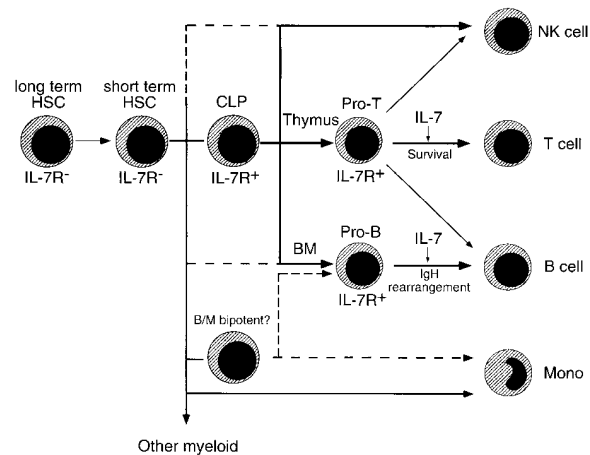

Figure 10. The Proposed Differentiation Sequences from HSCs to Lymphoid Cells in Adult Mice

The HSC population including both long-term and short-term HSCs does not express IL-7R. They might express IL-7R heterodimer immediately after the commitment to lymphoid lineages and become the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} CLPs that are able to generate all lymphoid classes. The CLPs differentiate into pro-B cells in bone marrow (BM), whereas the CLPs that successfully reach thymic microenvironment can read out T cell differentitation. The earliest thymic precursor (pro-T) population might include a small fraction of CLPs that have just homed to the thymus and still possess differentiation potential into B and NK cells (Wu et al., 1991a; Matsuzaki et al., 1993). IL-7R is expressed in both T cell and B cell progenitors, but IL-7 plays different roles in T- and B cell development in vivo; the principal role of IL-7 in T cell development is to maintain cell survival through up-regulating (at least) Bcl-2 (Akashi et al., 1997), but that in B cell development is other than maintenance of cell survival (Kondo et al., 1997). The principal role of IL-7 in B lymphopoiesis is probably to promote rearrangement of *IgH* genes (Corcoran et al., 1996). Some of lymphoid cells may be developed directly from short-term HSC or multipotent progenitors. It is still unclear that B cells can also be derived from bipotent B cell/myeloid progenitors (Cumano et al., 1992) in vivo (see Discussion).

the cells differentiate into morphologically determined dendritic cells after 7-day culture in methylcellulose containing SLF, IL-7, TNF-α, plus IL-1β (K. A. et al., unpublished data). On the other hand, the earliest thymic precursors did not give rise to colonies in the methylcellulose cultures containing IL-7, SLF, and/or FL (K. A. et al., unpublished data). The surface phenotype of the thymic precursor was different from that of the CLP population in the bone marrow (Figure 2). Furthermore, in contrast to the proven potency of the CLP cells in our competitive reconstitution assay (e.g., the 2 × 10^3 cells shown in Figure 2A), 4.5 × 10^3 thymic precursors from the same C57BL strain we used here gave rise to little or no progeny after intravenous injection to lethally irradiated congenic mice with 5 × 10^4 recipient-type bone marrow cells (Wu et al., 1991a), and the injection of large numbers (1–3 × 10^4) of cells was required to detect the B and NK cell progeny (Wu et al., 1991a; Matsuzaki et al., 1993). Thus, the thymic precursors and the CLP populations differ in surface phenotype, in frequency, in proven clonogenic capacity, and in their respective "burst" size. Based on these data, it is possible that the earliest thymic precursor population may be heterogeneous; this population may contain rare CLP

cells or a small number of oligopotent progenitors for B, NK, and/or LD cells as well as T cell–committed progenitors.

The IL-7R$^+$ CLP cells respond to IL-7 in cultures by expansion, at least for several cell doublings, retaining their lymphoid-specific differentiation capacity. Does the IL-7R play a role for generating these cells from more primitive HSC and/or progenitor cells? This seems unlikely, since the frequency of the phenotypically determined CLP population in γ_c-deficient mice is comparable to that in normal mice (M. K. et al., unpublished data). On the other hand, the γ_c-deficient mice showed reduced frequencies of early pro-B and pro-T cells (Kondo et al., 1997). IL-7R has been shown to mediate pivotal signals for promoting *IgH* rearrangement for B cell precursors (Corcoran et al., 1996) and survial for T cell precursors (Akashi et al., 1997). Thus, it could be that the expression of functional IL-7R complexes represents the initial cellular change in the differentiation from HSCs to CLP cells. It is important to clarify intracellular events linked to lymphoid commitment that lead to IL-7R expression. The expression of IL-7R may enable CLPs to respond to IL-7 in the context of local stromal microenvironments to give rise to the specified lymphocyte classes and subsets.

The generation of T and B cells from the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} CLP population is rapid compared to that from HSCs (Figures 5 and 6). Accordingly, it is reasonable to postulate that the CLP population from the bone marrow could represent a transition state between the known HSC (IL-7Rα^-) subsets (Spangrude et al., 1988; Morrison and Weissman, 1994; Osawa et al., 1996) and the earliest thymic precursors (Wu et al., 1991; Matsuzaki et al. 1993) that are IL-7Rα^+ (M. K. et al., unpublished data) (Figure 10). We have not yet critically assessed whether the Lin$^-$IL-7R$^+$Thy-1.1$^-$Sca-1^{lo}c-Kit^{lo} stage is a required intermediate for lymphoid differentiation. In the bone marrow, Antica et al. (1994) have reported that a Lin$^-$Thy-1^{lo}HSA$^+$Sca-2$^+$ population may be the latest precursor for generating the earliest thymic precursor. This population represented $\sim$0.25% of bone marrow cells ($\sim$10-fold more than our population) and retained pluripotent differentiation capacity. It is possible that T and B cell development may occur directly from some pluripotent progenitors or HSC subsets that induce clonogenic cells that retain the differentiation capacity to myeloid cells (Antica et al., 1994; Morrison and Weissman, 1994).

Several reports revealed a "close" relationship between the development of B and monocyte lineages: Klinken et al. (1988) demonstrated that the transfection of v-*raf* to B cell lines established from Eμ-*myc* transgenic mice could convert them into macrophages that maintained the identical rearrangement of immunoglobulin heavy chain (*IgH*) genes. The clonal analysis of human leukemias/lymphomas with B cell/myelomonocytic phenotypes (Mirro et al., 1986; Akashi et al., 1991; 1993) supports the possible existence of B cell/myelomonocytic bipotent progenitors, though the transformation event itself may perturb the normal differentiation process. In normal hematopoiesis, Hirayama et al. (1992) reported that B cells could develop from myeloid colony-forming cells from mouse bone marrow, but they later

showed that some of these cells retained the capacity for T cell differentiation (Hirayama and Ogawa, 1995), suggesting that the colony-forming cells might be pluripotent. A population of cells isolated from fetal liver gives rise to both B cells and macrophages from single cells in vitro (Cumano et al., 1992). However, T cell developmental potential was not tested in that study. It remains unclear whether B cells actually develop from "bipotent" B cell/myeloid or macrophage precursors in vivo.

Although the CLP population presented here has distinct phenotypes discernible from HSCs and completely lacks myeloid differentiation potential in a variety of assays both in vivo and in vitro, we should still take the limitation of the assays into account; the CLPs may not home suitable microenvironments for myeloid differentiation after intravenous injection in vivo or may be able to develop myeloid cells by the application of alternate, yet-to-be-developed in vitro assays. This warrants further analyses of the cellular characteristics of this population, including the expression of other known or unknown cytokine receptors and of transcription factors that are related to myeloid or lymphoid commitment. It is also of interest to determine the CLP population in fetal hematopoiesis to clarify fetal developmental processes of lymphoid cells including TCR/γ/δ T cells (Ikuta et al., 1990). Kawamoto et al. (1997) reported that T/B bipotent prognitors were undetectable in either the Lin$^-$c-Kit$^+$Sca-1$^+$ population enriched for HSCs or the Lin$^-$c-Kit$^+$Sca-1$^-$ progenitor fractions in fetal liver, determined by fetal thymic organ cultures of single cells in the presence of SLF, IL-3, and IL-7. However, results of lineage outcomes may reflect the bias of lineage readout in the assay system they used and/or the stochastic nature of lineage commitment of HSCs and progenitors. The isolation of a distinct population that possessed lymphoid-restricted differentiation potential in fetal liver will be a prerequisite for determining the potential role of CLPs in fetal lymphopoiesis.

The generation of T and B cells from the CLP population was prominent. The injection of as few as 10^3 CLPs could considerably restore the numbers of spleen B and T cells in the immunodeficient *RAG-2$^{-/-}$* mice as early as 2 weeks and 4 weeks after injection, respectively. Accordingly, the transplantation of the CLP population (or its human counterpart) might be able to rapidly correct the lymphoid deficiencies that are found in various immunocompromised situations. The transduction of genes into HSCs is currently difficult by using retrovirus vectors, because the long-term HSCs are mainly quiescent (Morrison and Weissman, 1994). Since a considerable fraction of CLPs is cycling and the CLPs could respond to defined cytokines in vitro, transduction of genes into the CLPs might be efficient, and this might benefit future gene therapy targeted for lymphoid cells.

Thus, our data provide direct evidence for the existence of a mouse clonogenic CLPs in vivo. Further characterization of this population could help clarify events in early lymphoid commitment. It is possible that a counterpart population in humans may also be isolated by using IL-7R as a marker as well as CD10, CD34, c-Kit, and/or Thy-1 (Galy et al., 1995). The identification of the

human CLPs might be useful for establishing lymphoid-restricted therapeutic approaches for various immune disorders.

Experimental Procedures

Mouse Strains

The congenic strains of mice, C57BL/Ka-Thy1.1 (Ly5.2) and C57BL/Ka-Thy1.1-Ly5.1 mice were used for the reconstitution assays. The strains differed only at the *Ly5* allele, and this difference made it possible to easily detect donor-derived cells generated from hematopoietic progenitors. C57BL/6 *RAG-2*$^{-/-}$ (Ly5.1) mice were generated by crossing C57BL/6 *RAG-2*$^{-/-}$ (Ly5.2) mice with C57BL/6-Ly5.1 mice. The C57BL/6 *RAG-2*$^{-/-}$ (Ly5.1) mice were used as recipients. Mice were bred and maintained in the animal care facility at Stanford University School of Medicine and were used at 4–10 weeks of age.

Cell Staining and Sorting

For sorting HSC and CLP populations, bone marrow cells were stained with unconjugated rat antibodies specific for lineage (Lin) markers (CD3, KT31.1; CD4, GK1.5; CD8, 53–6.7; B220, 6B2; Mac-1, M1/70; Gr-1, 8C5; and TER119). Lin$^+$ cells were partially removed with sheep anti-rat IgG-conjugated immunomagnetic beads (Dynabeads M-450, Dynal A. S., Oslo, Norway), and the remaining cells were stained with Cy5-PE (Tricolor)-conjugated goat anti-rat IgG polyclonal antibodies (Caltag, Burlingame, CA). After incubation with rat IgG (Sigma, St. Louis), cells were stained with FITC-conjugated anti-Thy-1.1 (19XE5), Texas red–conjugated anti-Sca-1 (E13-161-7) and APC-conjugated anti-c-Kit (2B8) monoclonal antibodies. IL-7Rα was stained with biotinylated anti-IL-7Rα antibody (A7R34) and was visualized by PE-conjugated streptavidin (Caltag). For the analysis of the pre-B and pro-B populations, bone marrow cells were stained with biotinylated anti-Gr-1 and TER119, PE-conjugated anti-CD43 (S7) (Pharmingen), Texas red–conjugated polyclonal anti-mouse IgM (Caltag), and APC-conjugated anti-c-Kit. Cells were then stained with Cy5-PE (RED670)–conjugated streptavidin (GIBCO BRL, Gaithersburg, MD). For the thymic progenitor population, thymocytes were stained with anti-CD4 and anti-CD8, followed by Texas red–conjugated anti-rat IgG polyclonal antibodies (Caltag). After incubation with rat IgG, cells were stained with PE-conjugated anti-CD3, APC-conjugated anti-c-Kit, and biotinylated anti-CD25 antibody (7D4, Pharmingen). Anti-CD25 was visualized by Cy5-PE-conjugated streptavidin. FITC-conjugated antibodies for HSA (CD24; M1/69), CD43, and CD44 (IM7) were purchased from Pharmingen. For cell cycle analysis, cells were first incubated with 10 μg/ml Hoechst 33342 (Sigma) as described previously (Morrison and Weissman, 1994). These cells were sorted or analyzed using a highly modified triple laser (488-nm argon laser, 599-nm dye laser, and UV laser) FACS (FACS Vantage, Becton Dickinson Immunocytometry Systems, Mountain View, CA). Progenitors were purified by sorting and then resorting to obtain precise numbers of cells that were essentially pure for the indicated surface marker phenotype. In the limit dilution assays and single-cell clonogenic assays, the resort was performed by using a carefully calibrated automatic cell deposition unit (ACDU) system (Becton Dickinson). This system deposited a specific number of purified cells onto either methylcellulose medium, S17 stromal cell cultures or HBSS in 96-well plates. In most cases, we reconfirmed the specific number of cells sorted in each well under an inverted microscope.

Evaluation of *TCR*β and *IgH* Gene Rearrangement Status

PCR analysis was used for detecting rearrangement of the *TCR*β and *IgH* genes as described previously (Mebius et al., 1997). Briefly, 2000 sorted cells were incubated at 94°C for 10 min in 0.5 % Tween D in PCR buffer prior to PCR reaction. Primer sequences used for *TCR*β gene in first PCR are 5′-TAGGCACCTGTGGGGAAGAAAC-3′ (Db2.1 ext) and 5′-TGAGAGCTGTCTCCTACTATC-3′ (Jb2.7 ext). Aliquots (1 of 5) were further amplified with internal primer pair, 5′-GTA TCACGATGTAACATTGTG-3′ (Db2.1 int) and 5′-GGAAGCGAGAGAT

GTGAATC-3′ (Jb2.7 int). The primers for detecting *IgH* rearrangement were 5′-GACTAAGCGGAGCACCACAG-3′ (DQ52) and 5′-CTCTCAGCCGGCTCCCTCAGGG-3′ (JH4#2). The samples were denatured (94°C, 30 s), annealed (58°C, 2 min), and extended (72°C, 3 min) for 35 cycles. Amplified DNA was subjected to electrophoresis in 1.2% agarose gel.

In Vivo and In Vitro Assays to Determine Differentiation Potential of CLPs

For reconstitution assays, the purified common lymphoid progenitors (CLPs) were injected into the retroorbital venous sinus of lethally irradiated (920 rad) congenic mice, which differed only at the *Ly5* allele, together with 2×10^5 host Ly5–type bone marrow cells. The levels of donor-derived blood nucleated cells were monitored as described elsewhere (Morrison and Weissman, 1994). When *RAG-2*$^{-/-}$ mice were used as recipients, purified cells were intravenously injected into *RAG-2*$^{-/-}$ mice after irradiation at 400 rad. Intrathymic injection was performed by directly injecting cells into thymuses of mice that had been irradiated (600 rad) as described previously (Akashi and Weissman, 1996; Akashi et al., 1997).

To support the formation of myeloid colonies, progenitors were cultured in an alpha-Modified Eagle Medium (αMEM)–based methylcellulose media (Methocult M3100; StemCell Technologies, Vancouver, Canada) that was supplemented with 30% fetal bovine serum (FBS), 1% bovine serum albumin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. Cytokines such as mouse SLF (100 ng/ml; provided by Immunex), mouse IL-3 (30 ng/ml; Genzyme, Cambridge, MA), mouse IL-6 (10 ng/ml; Genzyme), mouse GM-CSF (10 ng/ml; Genzyme), human M-CSF (25 U/ml; Genzyme), and human erythropoietin (1 U/ml) were added at the start of the culture. To examine lymphoid colony formation, we used Iscove's Modified Dulbecco's Medium (IMDM)–based Methocult M3630 (StemCell Technologies) containing human IL-7 (10 ng/ml) supplemented with SLF (100 ng/ml) and/or human FL (20 ng/ml; Genzyme). Progenitors were also cultured on irradiated (2000 rad)-S17 stromal cell layers in 96-well plates with RPMI 1640 medium containing 10% FBS (Gemini Bioproducts, Calabasas, CA), SLF (100 ng/ml), and human IL-7 (10 ng/ml; gift from Sanofi). All cultures were incubated at 37°C in a humidified chamber under 7% CO_2.

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