Immunity, Vol. 9, 827–838, December, 1998, Copyright ©1998 by Cell Press

Identification of a Fetal Hematopoietic Precursor with B Cell, T Cell, and Macrophage Potential

Georges Lacaud,* Leif Carlsson,† and Gordon Keller*‡§
*National Jewish Medical and Research Center
Department of Medicine
Denver, Colorado 80206
†Department of Microbiology
University of Umea
Umea 90187
Sweden
‡Department of Immunology
University of Colorado
Health Sciences Center
Denver, Colorado 80262

Summary

Despite years of investigation, precursor-progeny relationships within the developing lymphoid lineages of the hematopoietic system remain poorly defined. We have characterized the potential of precursors found within a subpopulation of fetal liver defined by AA4.1 and FcγRII/III expression and predominantly restricted to lymphoid and macrophage development. When cultured in methylcellulose with appropriate cytokines, AA4.1$^+$/FcγR$^+$ precursors generate colonies consisting of various lineages, including the combination of B cell, T cell, and macrophage. Retroviral marking studies showed that the lymphoid cells and macrophages within these colonies arise from a common precursor. These results demonstrate the presence of a common precursor with B cell-, T cell-, and macrophage-restricted potential and as such define an early restriction point within the fetal lymphoid system.

Introduction

The myeloid, erythroid, and lymphoid lineages of the hematopoietic system develop from multipotential stem cells through intermediate stage precursors that display various restricted patterns of commitment (Keller, 1992; Morrison et al., 1995). This pool of precursors represents a continuum that spans the entire developmental spectrum from immature multipotential cells to relatively mature lineage-restricted populations. Many of these precursors have been defined through the use of sensitive in vitro clonal assays that were initially established and optimized for the growth of erythroid and myeloid cells (Metcalf, 1977). Consequently, our understanding of the intermediate stages of development of these lineages is reasonably well advanced. In contrast, culture systems that promote the growth of B and T cell precursors have only recently been developed, and therefore the earliest stages of lymphopoiesis are not nearly as well defined as the early stages of myelopoiesis and erythropoiesis. However, with the identification of new cytokines, improvements in culture methods, and progress in cell separation techniques, significant advances in the isolation of novel lymphoid precursor populations have been made in recent years.

One important finding in this regard was the identification by Cumano et al. (1992) of a B cell/macrophage-restricted precursor in the AA4.1$^+$/Sca-1$^+$/B220$^-$/Mac-1$^-$ subpopulation of fetal liver. Although a number of earlier studies with immortalized lymphoid cell lines (Davidson et al., 1988; Borzillo et al., 1990; Principato et al., 1990) and transgenic mice (Klinken et al., 1988) suggested a close relationship between these lineages, the isolation of these bipotential precursors from fetal liver formally documented this unexpected myeloid/lymphoid branchpoint within the hematopoietic system. To date, T cell developmental potential has not been associated with this precursor. More recently, Kawamoto et al. (1997) provided further evidence for lymphoid and myeloid lineage combinations within the fetal lymphoid system. Analysis of the developmental potential of single Sca-1$^+$ fetal liver cells indicated the presence of restricted B cell/myeloid, T cell/myeloid, as well as multipotential B cell/T cell/myeloid precursors. Precursors with restricted B cell/T cell developmental potential were not identified in this analysis. Together, the studies on fetal liver suggest that the B and possibly the T cell lineages develop through intermediates that share a close relationship with the macrophage and possibly other myeloid lineages.

Further insights into lymphoid lineage relationships have been provided by the analysis of subpopulations of fetal and adult thymocytes. Although the large majority of cells within the thymus are of the T lineage, a number of different studies have demonstrated the presence of precursors from other lineages. For instance, at 12–14 days of gestation, the fetal thymus contains B cell, macrophage, and natural killer (NK) cell precursors in addition to those of the T lineage (Rodewald et al., 1992; Peault et al., 1994; Hattori et al., 1996; Carlyle et al., 1997). Studies on the adult thymus have identified populations that contain combinations of B cell, T cell, NK cell, and dendritic cell (DC) precursors (Wu et al., 1991; Ardavin et al., 1993; Godfrey and Zlotnik, 1993; Matsuzaki et al., 1993; Moore and Zlotnik, 1995; Wu et al., 1996). Erythroid and myeloid precursors were not detected in these populations. The presence of these different lineages within the thymus has led to the speculation that they develop from a common precursor, possibly the cell that seeds the organ in both fetal and adult life. While this interpretation is consistent with these findings, only the T cell and NK cell lineages have been linked to a common precursor by clonal analysis (Sanchez et al., 1994). The relationship of these other lineages within the thymus remains to be determined.

A somewhat clearer picture of the relationship between the NK, DC, and lymphoid lineages has begun to emerge from detailed analysis of human and mouse bone marrow subpopulations. Within the human hematopoietic system, Galy et al. (1995) identified a CD34$^+$/

§To whom correspondence should be addressed (e-mail: kellerg@njc.org).

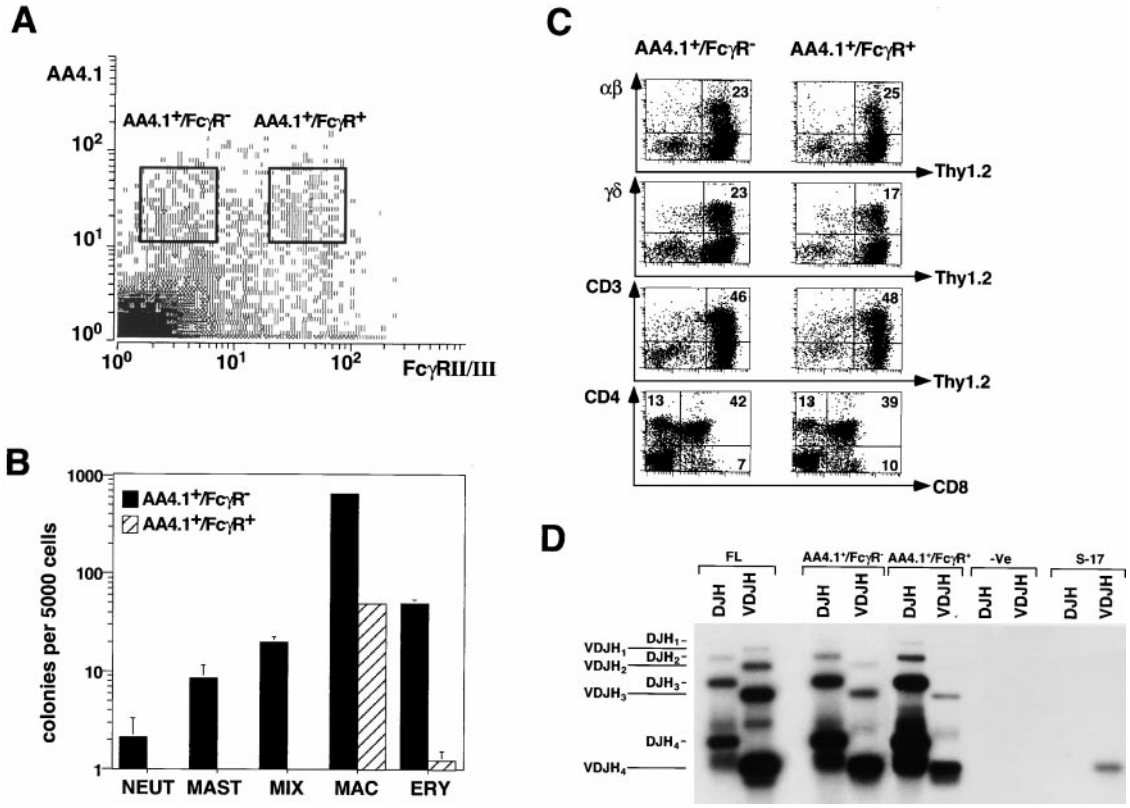

Figure 1. Characterization of the Myeloid/Erythroid and Lymphoid Potential of Day 13 Fetal Liver AA4.1$^+$/FcγR$^-$ and AA4.1$^+$/FcγR$^+$ Cells

(A) Representative FACS staining profile of fetal liver cells showing both the AA4.1$^+$/FcγR$^-$ and AA4.1$^+$/FcγR$^+$ populations. (B) Myeloid and erythroid precursor content of the AA4.1$^+$/FcγR$^-$ and AA4.1$^+$/FcγR$^+$ fractions. (C) Repopulation of fetal thymi in organ culture (FTOC) with freshly isolated day 13 fetal liver fractions. Host thymi express Thy 1.1., and fetal liver donor cells express Thy 1.2. Numbers in quadrants refer to the percentage of cells gated. (D) IgH rearrangements of fractionated fetal liver cells cultured on S-17 stromal cells in the presence of IL-7 for 2 weeks. The types of colonies scored are as follows: NEUT, neutrophil colonies; MAST, mast cell colonies; MIX, mixed colonies (i.e., colonies with an erythroid component plus at least two other lineages); MAC, macrophage colonies; ERY, erythroid colonies. Bars represent standard error of the mean of colony counts from 14 cultures. Absence of bars indicates that the error is too small to be visualized. FL represents day 13 total fetal liver, -Ve represents PCR reagents with no cells, and S-17 stromal cells (S-17) were used as the negative control.

CD10$^+$ fraction of bone marrow that contained a precursor with restricted B cell, NK cell, and DC potential. Whether or not this precursor also has T cell potential is unclear. In a more recent study, Kondo et al. (1997) identified a mouse bone marrow population that expresses the IL-7 receptor, low levels of Ly6A (Sca-1) and c-kit, and no detectable levels of lineage-specific markers. Functional analysis of this population demonstrated that it has B, T, and NK precursor activity but no detectable myeloid or erythroid potential. Limiting dilution studies indicated that some of the precursors within this population have both B and T cell potential, suggesting that a common lymphoid B/T precursor may exist in the bone marrow of the mouse.

Collectively, these studies have demonstrated the existence of several new lymphoid precursors and have provided evidence that suggests that others do exist. In addition, they have revealed several differences between lymphoid development in the adult and the fetus. To further define the earliest stages of fetal lymphoid development, we have characterized a subpopulation of fetal liver that expresses both AA4.1 and FcγRII/III and contains lymphoid and macrophage but few myeloid and erythroid precursors. By clonal analysis, we have identified a fetal AA4.1$^+$/FcγR$^+$ precursor with restricted B cell, T cell, and macrophage developmental potential. Given its potential, this precursor defines an early commitment step within the fetal hematopoietic system, one that is likely intermediate between the multipotential stem cell and the previously identified B/MAC precursor.

Results

Myeloid and Lymphoid Potential of Day 13 Fetal Liver Subpopulations

To study early steps of lymphoid commitment and to access early precursors, we focused our analysis on the onset of lymphopoiesis in the developing fetal liver. We have previously shown that day 12 fetal liver can be fractionated into distinct populations of lymphoid and myeloid precursors on the basis of expression of AA4.1 and the low-affinity Fc receptors, FcγRII/III (Carlsson et al., 1995). In the present series of experiments, we have

extended the analysis to day 13 of gestation as the liver contains significantly more cells than at day 12 but is still representative of early development. At day 12 of gestation, we found that only the fractions that expressed the AA4.1 marker, the AA4.1$^+$/FcγR$^-$ (hereafter referred to simply as AA4.1$^+$) and the AA4.1$^+$/FcγR$^+$ fractions, contained lymphoid precursors. Consequently, we have focused our efforts on the comparable fractions in day 13 fetal liver in this study. Figure 1A demonstrates that day 13 fetal liver contained the two expected AA4.1$^+$ fractions, each representing approximately 2%–4% of the total nucleated cell population.

The myeloid and erythroid potential of these two fractions was analyzed by colony growth in methylcellulose in the presence of a broad spectrum of hematopoietic cytokines. The AA4.1$^+$ fraction was enriched for precursors of multiple lineages, including neutrophil, mast cell, multipotential (mix), macrophage, and erythroid (Figure 1B), as observed previously with the comparable fraction from day 12 fetal liver (Carlsson et al., 1995). In contrast, the AA4.1$^+$/FcγR$^+$ subpopulation contained relatively few clonable precursors and those that were detected were predominantly restricted to the macrophage lineage. Occasionally a few erythroid, mast, and mix precursors were detected but their frequency was always low, often less than 1 per 5,000 cells. Analysis of the day 9 CFU-S content of the two fractions confirmed the findings from the in vitro colony assay. The AA4.1$^+$ population gave rise to an average of 27 spleen colonies (27.5 $\pm$ 2) per 2 $\times$ 10^4 cells injected, whereas the same number of AA4.1$^+$/FcγR$^+$ cells generated an average of less than one colony (0.7 $\pm$ 0.5) per mouse. Unfractionated fetal liver gave rise to 27 CFU-S (27.3 $\pm$ 2.5) per 10^5 cells, and uninjected controls contained no spleen colonies.

Lymphoid analysis demonstrated that both the AA4.1$^+$ and the AA4.1$^+$/FcγR$^+$ fractions contained T cell precursors that generated both $\alpha\beta$ and $\gamma\delta$ T cells in fetal thymi organ culture (FTOC, Figure 1C). Maturation of the lineages from these precursors appeared normal as demonstrated by the development of both CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells. When cultured on irradiated S-17 stromal cells in the presence of IL-7, cells from both fractions generated characteristic cobblestone areas that gave rise to B lymphocytes displaying both DJ_H and VDJ_H immunoglobulin heavy chain gene rearrangements (Figure 1D). Together, these data demonstrate that the AA4.1$^+$/FcγR$^+$ subpopulation from day 13 fetal liver contains B cell, T cell, and macrophage precursors but is depleted of precursors from the erythroid and other myeloid lineages. In contrast, the AA4.1$^+$ fraction contains erythroid and multiple myeloid precursors in addition to those of the B cell and T cell lineages.

Repopulating Potential of the AA4.1$^+$ and AA4.1$^+$/FcγR$^+$ Fetal Liver Fractions

To further evaluate the developmental potential of these fractions, varying numbers of cells from each were transplanted into irradiated SCID mice. Animals repopulated with 2 $\times$ 10^4 cells were analyzed at 4 weeks, while those that received 8 $\times$ 10^3 cells were sacrificed and analyzed 14 weeks following transplantation. At 4 weeks of repopulation, mice transplanted with either fraction contained donor-derived (H-2K^b) IgM$^+$ B cells in the peritoneal cavity and spleen and donor-derived CD3$^+$ T cells in the thymus (Figure 2A). In contrast to the lymphoid repopulation, striking differences in the myeloid/erythroid repopulation of the bone marrow of the two sets of mice were observed. Animals transplanted with the AA4.1$^+$ population contained significant numbers of myeloid (Mac-1$^+$ and Gr-1$^+$) and erythroid (TER-119$^+$) donor-derived cells in the marrow, demonstrating complete multilineage repopulation. In contrast, mice transplanted with AA4.1$^+$/FcγR$^+$ cells did not contain significant levels (above background) of donor myeloid and erythroid cells in the marrow, suggesting that, at this time point, reconstitution was primarily restricted to the lymphoid lineages. These findings are consistent with the in vitro analysis and further support the interpretation that the AA4.1$^+$/FcγR$^+$ fraction contains mostly lymphoid precursors but is devoid of intermediate-stage erythroid and myeloid precursors with the exception of those of the macrophage lineage. At week 14 of repopulation, mice transplanted with either AA4.1$^+$ or AA4.1$^+$/FcγR$^+$ cells again showed good lymphoid repopulation in all tissues analyzed. Analysis of the bone marrow compartments of mice transplanted with AA4.1$^+$/FcγR$^+$ cells indicated that they now contained detectable donor Mac-1$^+$, Gr-1$^+$, and TER-119$^+$ cells. Mice transplanted with AA4.1$^+$ cells continued to show almost complete marrow repopulation at this time point. The analysis of these long-term transplanted mice suggests that the AA4.1$^+$/FcγR$^+$ fraction does contain some stem cells that require more than 4 weeks to contribute significantly to the repopulation of these animals.

While the above study showed little myeloid/erythroid potential of the AA4.1$^+$/FcγR$^+$ population 4 weeks post transplantation, it is possible that this fraction contained more mature precursors that provided an early transient wave of repopulation. To investigate this possibility, the peripheral blood of mice transplanted with 1.5 $\times$ 10^4 AA4.1$^+$ or AA4.1$^+$/FcγR$^+$cells was analyzed within the first 4 weeks following cell injection. As shown in Figure 2B, mice transplanted with AA4.1$^+$ cells did contain significant levels of donor-derived IgM$^+$, Mac-1$^+$ and TER-119$^+$ cells in peripheral blood at 2 weeks following transplantation. In contrast, mice transplanted with the AA4.1$^+$/FcγR$^+$ fraction showed no repopulation in the peripheral blood at this time point (Figure 2B). After 6 weeks of reconstitution, the repopulation pattern of animals transplanted with these two populations was almost identical to that observed in the recipients in the experiments described in Figure 2A. Mice transplanted with AA4.1$^+$ cells showed multilineage repopulation, while those that received AA4.1$^+$/FcγR$^+$ cells showed predominantly lymphoid repopulation.

Taken together, the findings from the repopulation experiments are consistent with the interpretation that the AA4.1$^+$ fraction of fetal liver contains a broad spectrum of precursors ranging from multipotential stem cells to relatively later precursors that provide rapid repopulation. In contrast, the AA4.1$^+$/FcγR$^+$ fraction is enriched for B and T lymphoid precursors relative to myeloid and erythroid precursors and multipotential stem cells and as such represents an ideal population for the identification of possible novel precursors representing early stages of lymphoid development.

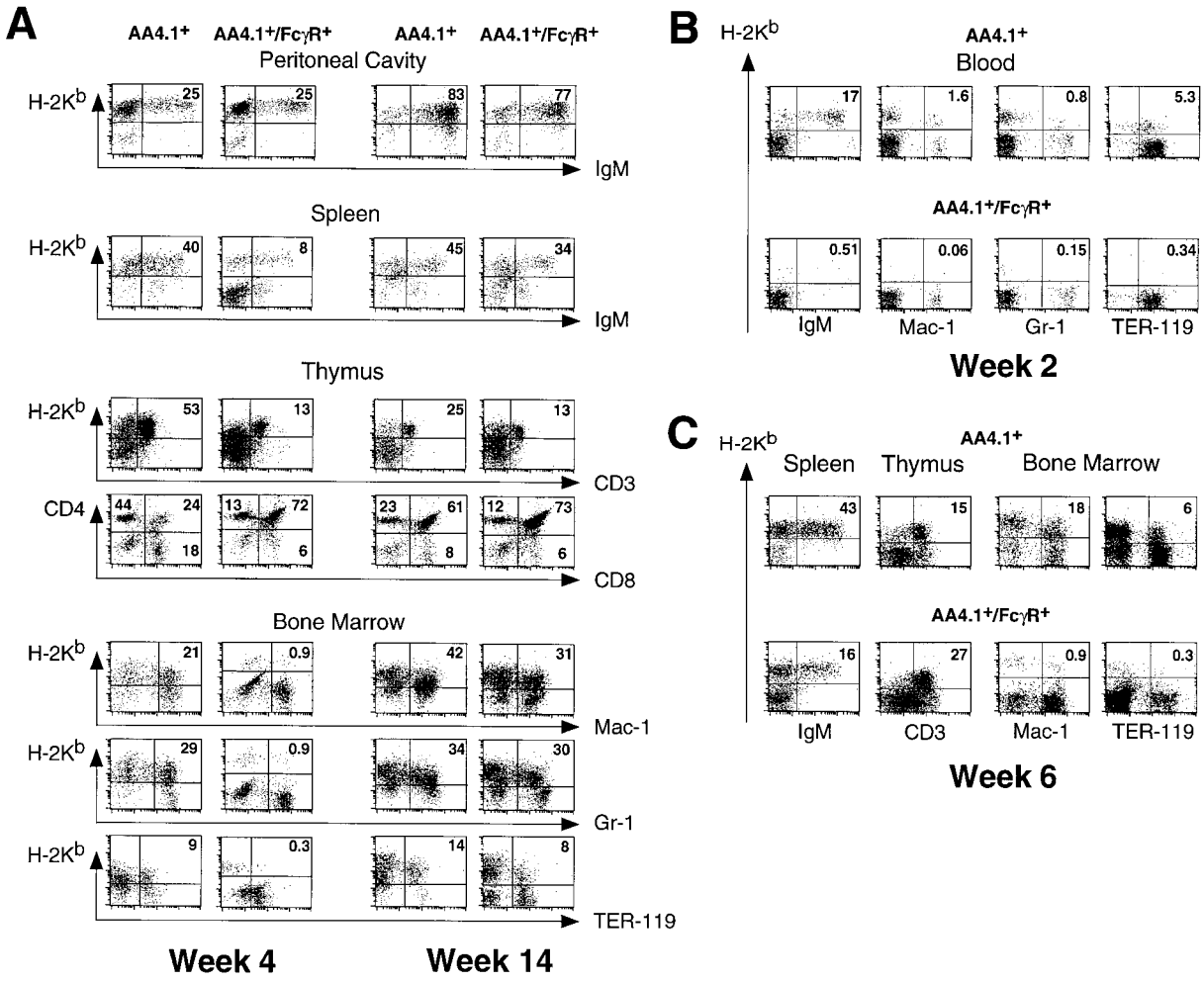

Figure 2. Analysis of SCID Mice Repopulated with Day 13 Fetal Liver AA4.1$^+$/FcγR$^-$ and AA4.1$^+$/FcγR$^+$ Cells

(A) Analysis of repopulation of SCID mice transplanted with varying numbers of AA4.1$^+$/FcγR$^-$ and AA4.1$^+$/FcγR$^+$ fetal liver cells. Animals transplanted with 2×10^4 cells were analyzed at 4 weeks, while those that received 8×10^3 cells were analyzed at 14 weeks following transplantation. Cells from peritoneal cavity, spleen, thymus, and bone marrow cells were stained with antibodies directed against H-2K^b or H-2K^d (data not shown) and different lineage markers. Donor C57BL/6 fetal liver cells express H-2K^b MHC, whereas the SCID recipients express H-2K^d. (B) Analysis of peripheral blood and (C) spleen, thymus, and bone marrow of SCID mice transplanted with 1.5×10^4 AA4.1$^+$/FcγR$^-$ and AA4.1$^+$/FcγR$^+$ fetal liver cells. Background staining in SCID mice is as follows: 0.2 $\pm$ 0.1% for H2K^b/IgM, 0.5 $\pm$ 0.3% for H2K^b/Mac-1, 0.3 $\pm$ 0.1% for H2K^b/Gr-1, and 0.8 $\pm$ 0.1% for H2K^b/TER-119 in peripheral blood, 0.8 $\pm$ 0.7% for H2K^b/IgM in spleen, 1 $\pm$ 0.3% for H2K^b/CD3 in thymus, 1.4 $\pm$ 0.6% for H2K^b/Mac-1, 0.9 $\pm$ 0.1% for H2K^b/Gr-1, and 0.9 $\pm$ 0.2% for H2K^b/TER-119 in bone marrow.

Colony Formation by d13 Fetal Liver AA4.1$^+$/FcγR$^+$ Precursors

To define the clonogenic potential of the lymphoid precursors in this fraction, AA4.1$^+$/FcγR$^+$ cells were cultured in methylcellulose in the presence of irradiated S-17 stromal cells and recombinant IL-7, conditions known to support the growth of lymphoid precursors in culture (Cumano et al., 1992). Under these conditions, macrophage colonies developed within the first 3–4 days of culture. Following an additional 3–4 days, a small proportion of these macrophage colonies showed further development, characterized by the outgrowth of small cells with a lymphoid morphology (Figures 3A and 3B). These colonies continued to grow over a 10–14 day culture period, reaching macroscopic sizes of greater than 10^5 cells. Similar colonies could be detected in cultures of AA4.1$^+$ cells. However, given the large numbers of other types of colonies in these cultures, their identification was difficult. Thus, most of the following analysis was done on colonies generated from AA4.1$^+$/FcγR$^+$ precursors. To define the lymphoid potential of these cells, colonies were picked from the methylcellulose cultures, pooled, and assayed for the presence of B cell precursors on S-17 stromal cells with IL-7 and for T cell precursors in FTOC. As shown in Figure 3C, the cells from pooled colonies did give rise to B cells showing DJ_H rearrangements and to both $\alpha\beta$ and $\gamma\delta$ T cells in the repopulated thymi.

In an effort to promote the development of all possible lineages within the colonies, S-17/IL-7 cultures were supplemented with interleukin-11 (IL-11), c-Kit ligand (KL), erythropoietin (Epo), and leptin. IL-11 and KL were

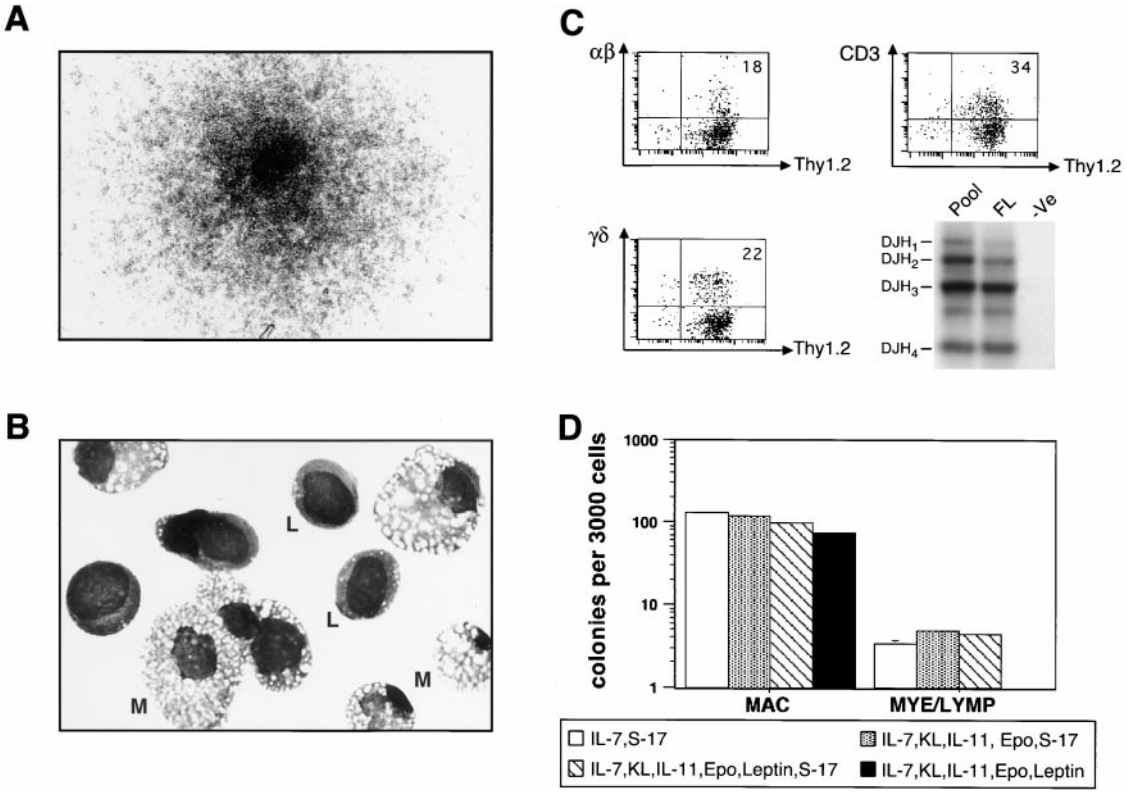

Figure 3. Morphology and Growth Factor Requirements of Myeloid/Lymphoid Colonies Generated from AA4.1$^+$/FcγR$^+$ Cells

(A) Morphology of a myeloid/lymphoid colony (original magnification 40×). (B) May-Grunwald Giemsa staining of the cells from a myeloid/lymphoid colony (original magnification 1000×). Cells with macrophage (M) and lymphoid (L) morphologies are indicated. (C) Analysis of the lymphoid potential of a pool of myeloid/lymphoid colonies grown in IL-7/S-17 conditions. FACS analysis of fetal thymi repopulated with cells from pooled colonies and DJ_H rearrangements of S-17 cultures of pooled colonies (Pool) and fresh fetal liver (FL) cells. (D) Frequency of macrophage (MAC) and myeloid/lymphoid colonies (MYE/LYMP) generated in different combinations of growth factors. Standard errors of the mean of the colony counts of 10 cultures are included but not visible in most cases.

included to support the development of early hematopoietic precursors. In addition, previous studies have shown that these cytokines together with IL-7 promote the development of myeloid/lymphoid colonies, including the B cell/macrophage precursor (Hirayama and Ogawa, 1994; Kee et al., 1994; Ball et al., 1995). Leptin was tested, as recent studies have suggested that it could play a role in lymphopoiesis (Baumann et al., 1996; Bennett et al., 1996; Cioffi et al., 1996; Gainsford et al., 1996). Epo was included to promote the differentiation of potential erythroid precursors within the colonies. To determine if these cytokines together with S-17 stromal cells do indeed support the development of both the myeloid and erythroid lineages, they were tested on the AA4.1$^+$ fraction. We used this fraction as a test population as it contains precursors for multiple lineages. From 5000 cells plated, the combination of IL-7, KL, IL-11, Epo, Leptin, and S-17 supported the development of 21 mast cell, 468 macrophage, 43 erythroid, and 30 multilineage colonies. In comparison, the myeloid/erythroid mix, used in our initial analysis (Figure 1B), supported the development of 11 mast, 470 macrophage, 45 erythroid, and 20 multilineage colonies in this experiment. To also determine if these conditions support the development and maintenance of precursors of multiple lineages able to generate secondary colonies, entire dishes were harvested and replated in secondary methylcellulose cultures. The equivalent of an entire primary culture established with 1500 AA4.1$^+$ cells replated on day 13 of culture generated an average of 200 neutrophil, 80 megakaryocyte, 1600 mast cell, 680 macrophage, and 450 erythroid secondary colonies. Together, these findings clearly demonstrate that these conditions do support the development of the erythroid and multiple myeloid lineages.

In the presence of IL-7, KL, IL-11, Epo, Leptin, and S-17, the myeloid/erythroid potential of the AA4.1$^+$/FcγR$^+$ fraction was identical to that observed in the previous analysis (Figure 1B), further confirming that this population was predominantly restricted to macrophage and lymphoid development. The number of myeloid/lymphoid colonies, generated from the AA4.1$^+$/FcγR$^+$ fraction, did not change dramatically with the addition of IL-11, KL, Epo, and leptin to S-17 and IL-7, and their development was still dependent on the presence of S-17 cells (Figure 3D). The additional cytokines did, however, promote the development of lineages other than macrophage and lymphoid in some of the myeloid/lymphoid colonies. Based on morphological assessment, approximately 10%–15% of these colonies were found to contain cells

Table 1. Developmental Potential of Myeloid/Lymphoid Colonies

Potential of Colonies	Combination of Growth Factors		
	IL-7, S-17 (37)	IL-7, KL, IL-11 Epo, S-17 (39)	IL-7, KL, IL-11, Epo Leptin, S-17 (29)
B/T/MAC/MAST/ERY	0% (0)	0% (0)*	14% (4)*
B/T/MAC	13% (5)	10% (4)*	45% (13)*
B/MAC	11% (4)	23% (9)	27% (8)
B	76% (28)	67% (26)*	14% (4)*

The combination of growth factors used and the total number of colonies with lymphoid potential detected (parentheses) are indicated in the column heading. Data are presented as percentage of lymphoid colonies with a given potential, and the corresponding number of colonies is indicated in parentheses. B, B cell; T, T cell; MAC, macrophage; MAST, mast cell; ERY, erythroid. Statistically significant differences ($p < 0.05$, Fisher Exact Test) in frequency of colonies with a given developmental potential generated in IL-7, KL, IL-11, Epo, and S-17 or IL-7, KL, IL-11, Epo, Leptin, and S-17 conditions are indicated by an asterisk.

of the erythroid, neutrophil, and mast cell lineages following more than 12 days of culture. Thus, under appropriate conditions, three major types of colonies were generated from the AA4.1$^+$/FcγR$^+$ cells; those consisting of pure macrophages, those containing macrophage and lymphoid cells, and those with a broader spectrum of lineage cells, including erythroid, neutrophil, mast cell, macrophage, and lymphoid.

B Cell, T Cell, and Myeloid/Erythroid Potential of Individual Colonies

To determine if B and T cell precursors resided within the same colony, individual myeloid/lymphoid colonies were picked and assayed as early as they could be distinguished from pure macrophage colonies (day 8–10 of culture). All of the colonies assayed had a visible macrophage component. Individual early colonies were fractionated and cells were tested for their ability to reconstitute FTOC, to give rise to B cells when seeded on S-17 in the presence of IL-7, and to grow and differentiate as myeloid/erythroid colonies in methylcellulose. Out of 41 single colonies analyzed from IL-7- and S-17-stimulated cultures, 37 contained lymphoid precursors, whereas the remaining 4 showed no precursor activity or displayed only myeloid/erythroid potential. As shown in Table 1, the majority (76%) of the colonies with lymphoid potential contained only B cell precursors (B) and displayed no T cell or myeloid/erythroid potential. Although all colonies contained a visible macrophage component when picked, many did not have detectable macrophage precursors suggesting a complete maturation of this lineage during colony growth under these conditions. A subpopulation of lymphoid colonies did retain some macrophage precursor potential as observed by the growth of secondary macrophage colonies in the replated cultures. Of these, approximately half also had B cell potential (B/MAC, 11%), whereas the remainder displayed the unique combination of B cell/T cell potential (B/T/MAC, 13%). No colonies with T cell/macrophage (T/MAC)-, B cell/T cell (B/T)-, or T cell (T)-restricted potential were detected.

When grown in the presence of IL-7, KL, IL-11, Epo, and S-17, the majority of the 39 colonies with lymphoid potential analyzed contained only B lineage precursors, as in the previous conditions. These additional cytokines did increase the frequency of the B/MAC colonies but had no effect on the frequency of colonies that contained B, T, and MAC precursors. With the addition of leptin to this combination of cytokines, the potential of the colonies changed dramatically. Almost half of the myeloid/lymphoid colonies were found to contain B cell, T cell, and macrophage precursors (45%). Furthermore, 14% of the colonies generated under these conditions contained erythroid, mast, and macrophage precursors in addition to those of the B cell and T cell lymphoid lineages (B/T/MAC/MAST/ERY). This frequency is in agreement with the previous morphological analysis, which indicated that approximately 10%–15% of myeloid/lymphoid colonies generated under these conditions contained erythroid and multiple myeloid lineages. Finally, in the presence of leptin, the proportion of colonies displaying only B cell precursor potential was significantly reduced. Representative results demonstrating the presence of B and T cell precursors in a B/T/MAC/MAST/ERY colony (colony 15) and in a B/T/MAC colony (colony 16) are shown in Figures 4A and 4B. Taken together, these findings demonstrate that leptin, in combination with IL-7, KL, IL-11, Epo, and S-17, supports the efficient development of myeloid, erythroid, and especially T cell precursors within the colonies. The fact that all types of colonies, B/T/MAC/MAST/ERY, B/T/MAC, B/MAC, and MAC, developed in these culture conditions strongly suggests that the restricted patterns of lineage development within the colonies reflect the presence of precursors with distinct potentials.

Gene Expression Analysis of the Myeloid/Lymphoid Colonies

Based on precursor analysis, the previous set of experiments demonstrated differences in the developmental potential of the myeloid/lymphoid colonies. While this approach has the capacity of measuring rare populations from multiple lineages, it can be somewhat misleading if the lineage of interest has matured beyond the clonogenic stage. Thus, to further characterize the developmental potential of the myeloid/lymphoid populations, individual day 8–10 colonies were analyzed in parallel for gene expression pattern and lineage potential. As shown in Figure 5, all the colonies displaying either B/T/MAC/MAST/ERY, B/T/MAC, B/MAC, or B lineage potential expressed *c-fms*, a macrophage-specific gene that encodes the receptor to M-CSF. Expression

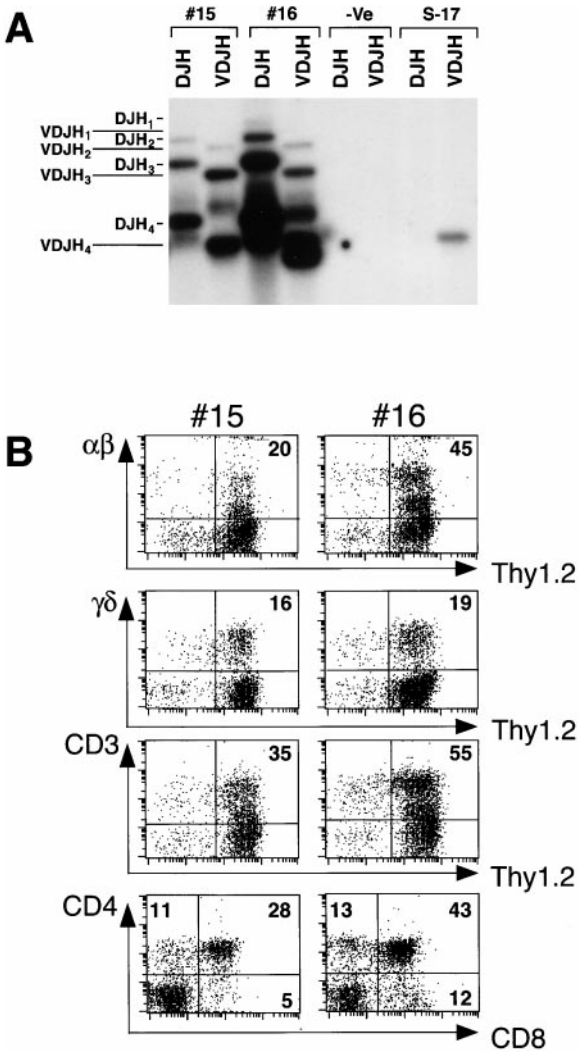

Figure 4. Analysis of B Cell and T cell Precursor Content of Individual Colonies

(A) IgH rearrangements of cells from colonies with either B/T/MAC/MAST/ERY (#15) or B/T/MAC (#16) developmental potential. (B) FACS analysis of fetal thymi repopulated with cells from colonies #15 and #16.

of *c-fms* in all colonies is consistent with the fact that they all contained initially a macrophage component, including those that only displayed B cell and no macrophage precursor activity upon replating (depicted as B colonies in Figure 5). The transcription factor Ikaros (Georgopoulos et al., 1994) and the Ig heavy chain Cμ genes were also expressed in all colonies, confirming the functional studies that demonstrated that they contained either B cell and T cell or only B cell precursors. In contrast to the broad expression pattern of these genes, GATA-1, a transcription factor specific to the mast cell, erythroid, and megakaryocyte lineages (Orkin, 1992), was only detected in the colonies that contained precursors for these lineages (B/T/MAC/MAST/ERY). The absence of GATA-1 expression in the B/T/MAC, B/MAC, and B colonies confirms the precursor analysis in establishing that they do not contain cells of these lineages. The lack of β major globin expression in the B/T/MAC/MAST/ERY colony suggests that the erythroid lineage is immature and has not yet undergone significant hemoglobinization.

Clonal Analysis of the Myeloid/Lymphoid Colonies

The previous data suggest the existence of a precursor with B cell, T cell, and macrophage potential, assuming that the individual colonies are clonal. To address this issue, we used a retroviral marking method to track the origin of the lineages within these colonies. Freshly isolated AA4.1$^+$/FcR$^+$ cells were infected with N2, a recombinant retrovirus that expresses the neo^r gene, by coculture with the virus-producing cell line. After infection, the fetal liver cells and virus-producing cells were harvested by trypsinization and seeded at low density in methylcellulose in the presence of IL-7, KL, IL-11, Epo, Leptin, S-17, and G418. Individual G418-resistant myeloid/lymphoid colonies were picked and B cells, T cells, macrophages, and in some experiments mast cells generated from them. An aliquot of each colony was also assayed for myeloid and erythroid potential. Out of 54 individual neo^r colonies, 12 (22%) displayed B/T/MAC potential, whereas 3 (5%) contained the multilineage combination of erythroid, mast cell, macrophage, B, and T lymphocyte precursors (B/T/MAC/MAST/ERY). As the number of cells available to characterize the retroviral integration sites was limited, a sensitive inverse PCR method was utilized for this analysis. With this approach, a small fragment of DNA adjacent to the 3′ LTR of the retrovirus was amplified, visualized on an agarose gel, and subsequently sequenced (Figure 6A). A total of 13 colonies were analyzed, 11 with B/T/MAC potential and 2 with B/T/MAC/MAST/ERY potential. A common band in the B cell, T cell, and macrophage lineages from eight individual B/T/MAC colonies was successfully amplified. Sequence analysis indicated that the common band in each of the lineages from an individual colony was identical, demonstrating a common integration site. In addition, the sequence analysis clearly showed distinct integration sites between different colonies. These results demonstrate that the B cell, T cell, and macrophage lineages within a colony develop from a common precursor. Representative analysis of four of these colonies is presented in Figures 6B and 6C. Similarly, B cells, T cells, and macrophages from one of the colonies with B/T/MAC/MAST/ERY potential, and B cells, T cells, macrophages, and mast cells from the second of these colonies were found to contain the same retroviral integration site, demonstrating that these colonies were also clonal (data not shown). More restricted bands were also observed in this analysis, possibly reflecting secondary infection during colony growth of the AA4.1$^+$/FcγR$^+$ precursors, as virus-producing cells were harvested and included in the methylcellulose with fetal liver cells. Thus, the MAC/B cell–restricted band observed in colony 2 and 3 and the T cell–restricted band in colony 4 of Figure 6B may reflect further commitment to a B/MAC or T cell precursor during colony growth.

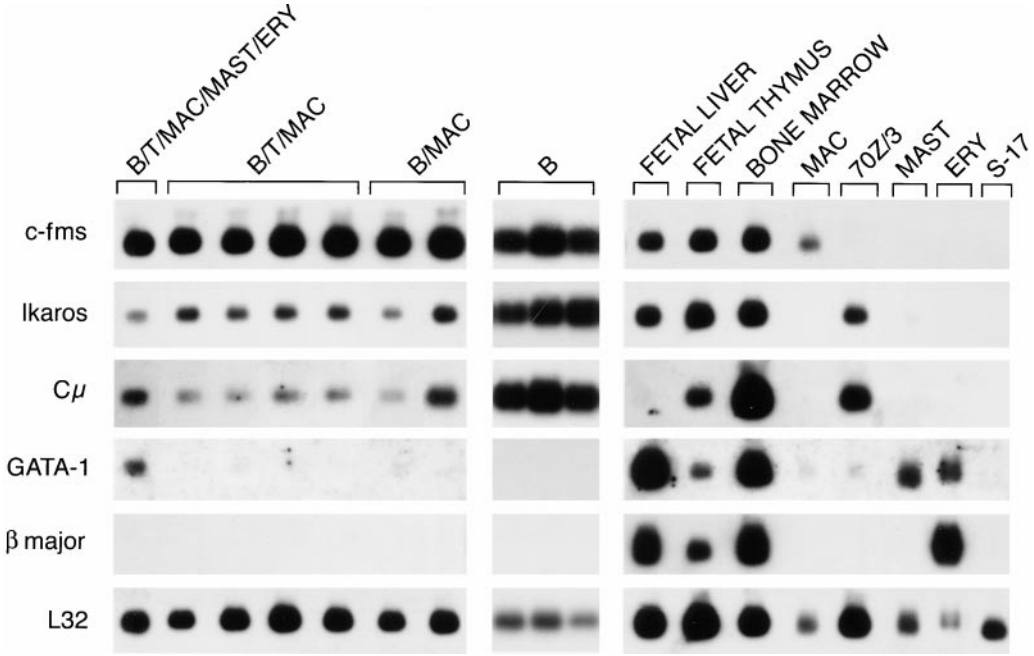

Figure 5. Gene Expression Analysis of Myeloid/Lymphoid Colonies

Aliquots of colonies were amplified by polyA$^+$ PCR and hybridized with the 3′ fragment of the cDNA of the indicated genes. Colonies with functional B/T/MAC/MAST/ERY, B/T/MAC, B/MAC, or B developmental potential were analyzed. Controls include unfractionated day 13 fetal liver cells, day 15 fetal thymocytes, bone marrow cells, macrophage colonies from fetal liver (MAC), a pre-B cell line (70Z/3), mast cell colonies from fetal liver (MAST), erythroid colonies from bone marrow (ERY), and the S-17 stromal cells (S-17).

Discussion

The findings presented in this report demonstrate the existence of a precursor with B cell, T cell, and macrophage potential in the early stages of fetal liver development. This B/T/MAC precursor may represent one of the earliest lymphoid restriction points within the fetal hematopoietic system. These findings extend the observations of Cumano et al. (1992) and demonstrate that, in addition to the B cell lineage, the fetal T cell lineage also develops in close association with the macrophage lineage. While we did not detect any T/MAC-restricted cells, the recent study of Kawamoto et al. (1997) suggests that this type of precursor may exist as well. Taken together, these observations provide strong evidence that the fetal lymphoid system develops from a lymphoid/macrophage-restricted precursor.

Although the evidence for a lymphoid/macrophage-restricted precursor in the fetal system is solid, the relationship between these lineages in the adult lymphoid system is less well defined. A number of different studies using adult bone marrow or spleen populations have demonstrated "lineage switching" from B cells to macrophages suggesting a close relationship between these lineages in the adult (Davidson et al., 1988; Klinken et al., 1988; Borzillo et al., 1990; Principato et al., 1990; Borrello and Phipps, 1996). However, the equivalent of the B/MAC or the B/T/MAC has not yet been identified in the bone marrow. The concept of an adult lymphoid/macrophage-restricted precursor has been challenged by the recent studies of Kondo et al. (1997), which described a common lymphoid precursor (CLP) with B cell and T cell potential but lacking the capacity to generate detectable myeloid or erythroid progeny. These apparent differences between fetal and adult lymphopoiesis could reflect fundamental differences in the development of the two systems or the fact that the adult B/T/MAC and B/MAC and the fetal CLP have not yet been identified. Future experiments will distinguish between these possibilities.

The full developmental potential of the B/T/MAC precursor may extend beyond B cells, T cells, and macrophages, as there is mounting evidence that suggests that NK cells and possibly a subpopulation of dendritic cells are closely related to the lymphoid lineages (Lanier et al., 1992; Rodewald et al., 1992; Ardavin et al., 1993; Sanchez et al., 1994; Wu et al., 1996; Carlyle et al., 1997; Wu et al., 1997). Preliminary studies have indicated that the myeloid/lymphoid colonies do contain NK1.1$^+$ cells, suggesting that the B/T/MAC precursor does have NK potential (unpublished data). Functional analysis will be required to determine if these cells are indeed NK cells. The dendritic cell potential of the B/T/MAC remains to be determined. A precursor with B/T/MAC/NK/DC potential could represent the cell that seeds the fetal thymic rudiment, as studies have demonstrated the presence of these lineages in this developing organ. Our current studies aimed at isolating and characterizing early thymic precursor populations will determine if the equivalent of the fetal liver B/T/MAC exists in the fetal thymus.

Analysis of the growth factor requirements of the B/T/MAC precursors revealed a potential role for leptin in early lymphopoiesis. The fact that leptin did not increase the number of myeloid/lymphoid colonies suggests that

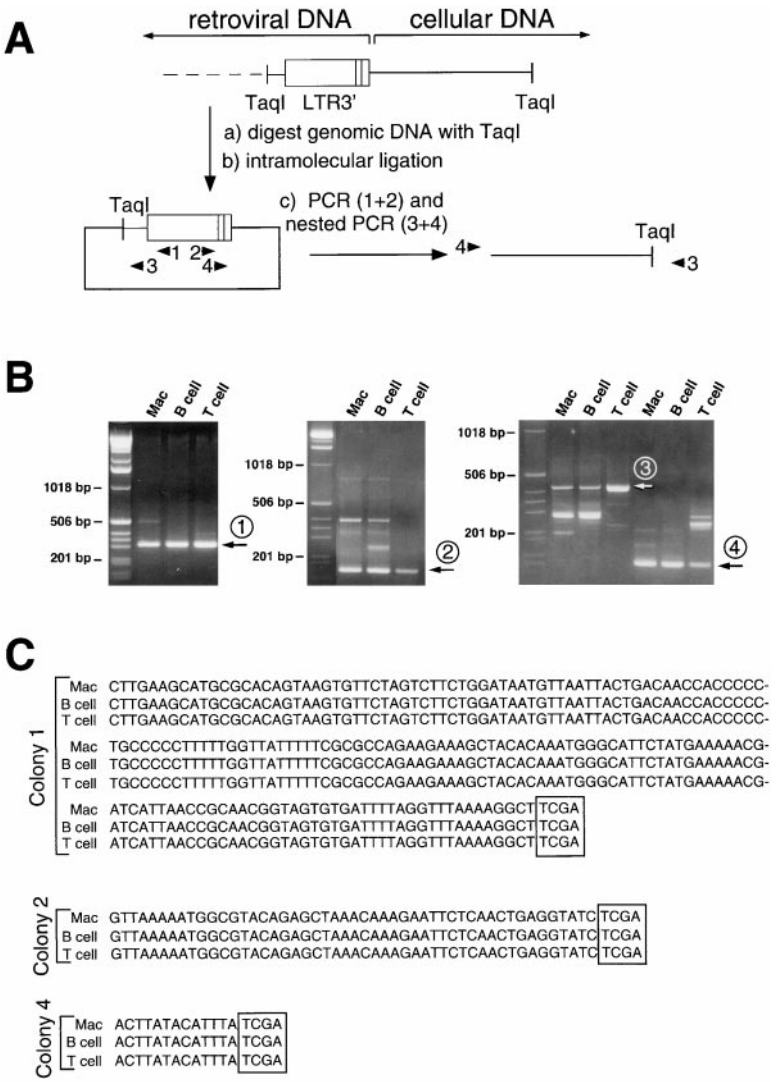

Figure 6. Clonal Analysis of the B Cell, T Cell, and Macrophage Precursors in the Myeloid/Lymphoid Colonies

(A) Scheme of the inverse PCR strategy used for identification of the retroviral integration sites in the different lineages. 1, 2, 3, and 4 refer to the oligonucleotides used in this analysis. (B) Ethidium bromide–stained gels of the PCR products from macrophages, B cells, and T cells generated from four individual G418-resistant colonies. Arrows show common bands in the three different lineages. (C) Sequences of the cellular DNA found in the common bands shown in 6B. Sequence from macrophage, B cell, and T cell populations derived from the colonies 1, 2, and 4 are shown. TaqI restriction site sequences are indicated by boxes.

it does not stimulate the development of B/T/MAC precursor but rather promotes the development/survival of lymphoid and myeloid precursors within these colonies. Our findings are consistent with previous studies that have demonstrated that leptin can promote the growth of myeloid, erythroid, and lymphoid precursors in culture and that the leptin receptor is expressed in various hematopoietic and lymphoid precursor populations (Baumann et al., 1996; Bennett et al., 1996; Gainsford et al., 1996; Mikhail et al., 1997). In addition, it has been shown that *db/db* mutant mice that express a truncated splice variant of the leptin receptor have reduced numbers of early B cell precursors as well as reduced numbers of lymphoid and myeloid colony forming cells compared to wild-type animals (Bennett et al., 1996; Chen et al., 1996; Lee et al., 1996). While leptin did enhance the potential of the fetal liver-derived myeloid/lymphoid colonies, preliminary studies demonstrate only low levels of leptin receptor in these colonies (unpublished data). In contrast, S-17 stromal cells appear to express abundant levels of the receptor, suggesting that the effects of leptin could be indirect. Future studies will be required to determine if this is indeed the case.

The findings from the in vitro and in vivo experiments indicate important differences between the AA4.1$^+$ and the AA4.1$^+$/FcγR$^+$ fetal liver fractions. Specifically, expression of FcγR$^+$ together with AA4.1 appears to define a subpopulation enriched for precursors committed to lymphoid and macrophage development. These findings together with previous studies showing FcγR$^+$ expression on fetal liver B cell precursors (Carlsson et al., 1995) and fetal thymic T cell and NK cell precursors (Rodewald et al., 1992; Hattori et al., 1996) suggest that this family of receptors could play some role in early lymphoid development. While the expression data are consistent with this interpretation, analysis of FcR γ chain knockout mice that do not express FcγRIII did not reveal any significant alterations in adult lymphoid development (Takai et al., 1994). Thus, the function of FcγR$^+$ on these subpopulations of lymphoid precursors remains to be determined.

In conclusion, we have identified a precursor with the potential to generate B cells, T cells, and macrophages in a subpopulation of day 13 fetal liver defined by the expression of AA4.1 and FcγRII/III. The developmental potential of this precursor defines an early restriction

point during the establishment of the lymphoid system that links the fetal B cell and T cell lineages at a stage beyond the multipotential stem cell. Access to the colonies generated by this precursor will provide an opportunity to further define the molecular events involved in the development of these lymphoid lineages.

Experimental Procedures

Mice

C57BL/6 mice (Taconic) were paired late in the afternoon and the females checked the next morning for the presence of a vaginal plug (day 0). Pregnant mice were sacrificed 13 days later, the uteri removed, and the livers harvested from the fetuses. Fetal thymi were obtained from day 15 timed mated outbred Swiss/Webster mice (Taconic). CB17 SCID mice were obtained from a breeding colony maintained in the animal care facility of the National Jewish Medical and Research Center. Before transplantation, SCID mice were pretreated with an intraperitoneal injection of 50 μl of reconstituted anti-asiolo GM1 (Wako Pure Chemical Industries) and sublethally irradiated with 300 rads from a 137Cs source. Anti-asiolo GM1 treatments were repeated on days 5 and 11 following injection of the fetal liver cells via the tail vein.

CFU-S Assay

Different fetal liver cell populations were transplanted via tail vein injection into irradiated (920 rads) C57BL/6 recipient mice. Nine days following transplantation, the mice were sacrificed, their spleens fixed in Bouins solution, and the colonies scored under a dissecting microscope.

Cell Sorting, Staining, and Antibodies

Fetal liver cells were stained as previously described (Carlsson et al., 1995). Between 40–70 $\times$ 10^6 stained cells were sorted on an Epics Coulter 741 or a Coulter Elite cell sorter. Reanalysis of the sorted AA4.1$^+$ and AA4.1$^+$/FcγR$^+$ cells revealed a purity of $>$90%. For analysis of reconstituted mice or cells from fetal thymic organ cultures, single cell suspensions were preincubated with unlabeled 2.4G2 antibody to prevent nonspecific binding to the FcR. Antibodies used for staining included biotinylated EH144.19 (H-2K^b), biotinylated 34.5.8 (H-2K^d), biotinylated 536.7212 (CD8), FITC-labeled OX-7 (Thy-1.1, Pharmingen), FITC-labeled 53-2.1 (Thy-1.2, Pharmingen), FITC anti-IgM (Southern Biotechnology Associates, Inc), FITC-labeled 145-2C11 (CD3), FITC-labeled M1/70 (Mac-1, Pharmingen), FITC-RB6-8C5 (Gr-1, Pharmingen), PE-labeled H57-597 ($\alpha\beta$ TCR, Pharmingen), PE-labeled GL4 ($\gamma\delta$ TCR, Pharmingen), PE-labeled H129.19 (CD4, Pharmingen), and PE-TER-119 (Pharmingen).

Clonal Assays

The colony assay for detection of myeloid and erythroid precursors was carried out in Petri dishes in 1 ml cultures containing 1% methylcellulose in Iscoves Modified Dulbecco's Medium (IMDM, GIBCO), 10% plasma-derived serum (PDS; Antech, TX) and the following mixture of growth factors: interleukin-1 ([IL-1] 1000 units/ml), interleukin-3 ([IL-3] 1% conditioned medium), kit ligand ([KL] 1% conditioned medium), interleukin-11 ([IL-11] 35 ng/ml), erythropoietin ([Epo] 2 units/ml), macrophage-colony stimulating factor ([M-CSF] 100 units/ml), granulocyte-macrophage-colony stimulating factor ([GM-CSF] 20 units/ml), and thrombopoietin ([TPO] 5 ng/ml). Recombinant human IL-1α was kindly provided by Hoffmann-La Roche (Nutley, NJ), IL-3 was provided by conditioned medium (CM) of X63 Ag8–653 myeloma cells transfected with a vector expressing IL-3 (Karasuyama and Melchers, 1988), and KL was provided by CM of a cell line transfected with a vector expressing KL kindly provided by Genetics Institute. Recombinant M-CSF, IL-11, and GM-CSF, TPO, were purchased from R&D Systems. All factors were tested individually to give optimal growth. All single lineage colonies were scored at days 7–9, while the multilineage colonies were scored at days 9–14.

Myeloid/lymphoid colonies were grown from freshly isolated fetal liver cells in 1 ml cultures containing 1% methylcellulose in IMDM, 10% fetal calf serum (FCS), γ-irradiated S-17 stromal cells (10^5/ml), and different combinations of growth factors. Recombinant IL-7 (Upstate Biotechnology, Inc) and leptin (PrepoTech) were used at 16 ng/ml and 250 ng/ml, respectively. Different batches of leptin were used with similar results. For developmental analysis, individual colonies were picked following 8 to 10 days of culture and fractionated as follows: 20% of the cells for the myeloid/erythroid precursor assay, 10% of the cells for the B cell precursor assay, and the remaining 70% of the cells for the T cell precursors assay. In some experiments, 10% of the cells of a colony were used for gene expression analysis and 60% of the cells assayed for T cell precursors in FTOC.

B Cell Assay and Amplification of IgH Gene Rearrangements from Genomic DNA

Freshly isolated fetal liver fractions or cells from myeloid/lymphoid colonies were cultured on irradiated S-17 stromal cells in IMDM supplemented with 10% FCS and 3% IL-7 CM from NIH 3T3 cells transfected with a vector expressing IL-7 (Rolink et al., 1991). Following 1 to 2 weeks of culture, the cells were harvested and the cellular DNA analyzed for Ig heavy chain gene rearrangements by PCR as previously described (Carlsson et al., 1995). Hybridizations were performed with a XbaI-HindIII germline J_H4 probe. The V_H PCR oligonucleotide also primed between the J_H3 and J_H4 germline genes giving rise to a band of approximately the same size as a VDJ_H4 rearrangement. This band hybridized to the J_H4 containing probe and was used as an internal control for the PCR reaction and hybridization.

Fetal Thymic Organ Cultures

Thymus lobes were dissected from outbred Swiss/Webster fetuses at day 15 of gestation and seeded with fetal liver cells as previously described (Carlsson et al., 1995). After 2 to 3 weeks of culture, the thymic lobes were harvested, the cells dissociated, blocked with unlabeled 2.4G2, and stained with appropriate antibodies. Cells of fetal liver origin were identified by the presence of the Thy 1.2 allele and could be distinguished from cells of fetal thymi origin that express Thy 1.1.

Gene Expression

Gene expression was determined using the polyA$^+$ PCR method described by Brady et al. (1990). Reverse transcription, tailing, and PCR procedures were performed as described, with the exception that the (dT)-x oligonucleotide was shortened to 5′-CATCTCGAGGG CCGC$(T)_{24}$-3′. Amplified products from the PCR reaction were separated on agarose gel and transferred to a Z-probe GT membrane (Biorad). The resulting blots were hybridized with ^{32}P randomly primed cDNA fragments corresponding to the 3′ region of the genes.

Infection of Precursors and Analysis of Retroviral Integration

Freshly isolated d13 FL AA4.1$^+$/FcR$^+$ cells were infected by overnight coculture with γ-irradiated GPE86-N2 producing cells in the presence of IL-7, KL, IL-11, Epo, Leptin, polybrene (4 μg/ml), and 10% FCS in IMDM. The following day, cells were harvested by trypsinization and plated at low density in methylcellulose in the presence of IL-7, KL, IL-11, Epo, Leptin, γ-irradiated S-17 stromal cells, and G418 (1 mg/ml). After 8 to 10 days of culture, individual myeloid/lymphoid colonies were picked and fractionated to grow different cell populations. Macrophages were grown on tissue culture dishes in IMDM containing 30% L cell CM, IL-3, and 10% FCS. After 2 days, the cells were passaged into 30% L cell CM, 10% FCS, and IMDM. The cells were subsequently maintained and passaged into the same media for 2 to 3 weeks to obtain a homogeneous monolayer of macrophages. B cells were grown on irradiated S-17 in the presence of IL-7. The cultures were passaged 2 to 3 times. Mast cells were grown in bacterial grade dishes in IMDM media containing KL, IL-3, and 10% FCS. The suspension cells were frequently passaged to eliminate adherent cells and to generate a homogeneous population of mast cells. T cells were harvested from reconstituted fetal thymi.

Genomic DNA was digested overnight with TaqI and then phenol/chloroform extracted and precipitated. An aliquot of diluted DNA was ligated, and the reaction was stopped by phenol/chloroform extraction. Primer sequences used in the first PCR are 5′-AGGACCT

GCTTACCACA-3′ (oligonucleotide 1) and 5′-CTGTTCCTTGGGAG GGT-3′ (oligonucleotide 2). The samples were denatured (95°C, 1 min), annealed (40°C, 1 min), and elongated (72°C, 1 min) for 35 cycles. Aliquots of 1/10 of these reactions were further amplified with the following nested primers: 5′-GCTGGTGATATTGTTGAGTCA AAAG-3′ (oligonucleotide 3) and 5′-TTGGGAGGGTCTCCTCT GAG-3′ (oligonucleotide 4). The samples were denatured (95°C, 1 min), annealed (48°C, 1 min), and elongated (72°C, 2 min) for 35 cycles. The resulting PCR products were separated on a 1.5% agarose gel, and DNA from common bands were purified with QIAEX II resin (Qiagen) and sequenced using oligonucleotide 4 as primer.

Acknowledgments

We wish to thank W. Townend and S. Sobus for providing expert assistance with the cell sorting, A. Shafer and J. Cioffi (Progenitor Inc) for the analysis of leptin receptor expression, L. Landskroner for assistance with graphics, and A. Grigoriadis, V. Kouskoff, R. Lapadat, and S. Robertson for critical comments on this manuscript. G. L. was a recipient of the Melvin Garb Foundation endowed fellowship in Basic Immunology. This work was supported by a grant from the American Cancer Society (#DHP-152) and in part by a National Institutes of Health PO1 AI22295 grant.

Received March 24, 1998; revised October 19, 1998.

References

Ardavin, C., Li, C.L., and Shortman, K. (1993). Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. Nature *362*, 761–763.

Ball, T.C., Hirayama, F., and Ogawa, M. (1995). Lymphohematopoietic progenitors of normal mice. Blood *85*, 3086–3092.

Baumann, H., Morella, K.K., White, D.W., Dembski, M., Bailon, P.S., Kim, H., Lai, C.F., and Tartaglia, L.A. (1996). The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. Proc. Natl. Acad. Sci. USA *93*, 8374–8378.

Bennett, B.D., Solar, G.P., Yuan, J.Q., Mathias, J., Thomas, G.R., and Matthews, W. (1996). A role for leptin and its cognate receptor in hematopoiesis. Curr. Biol. *6*, 1170–1180.

Borrello, M.A., and Phipps, R.P. (1996). The B/macrophage cell: an elusive link between CD5+ B lymphocytes and macrophages. Immunol. Today *17*, 471–475.

Borzillo, G., Ashmun, R., and Sherr, C. (1990). Macrophage lineage switching of murine early pre-B lymphoid cells expressing transduced *fms* genes. Mol. Cell. Biol. *10*, 2703–2714.

Brady, G., Barbara, M., and Iscove, N.N. (1990). Representative *in vitro* cDNA amplification from individual hemopoietic cells and colonies. Methods Mol. Cell. Biol. *2*, 17–25.

Carlsson, L., Candeias, S., Staerz, U., and Keller, G. (1995). Expression of Fc gamma RIII defines distinct subpopulations of fetal liver B cell and myeloid precursors. Eur. J. Immunol. *25*, 2308–2317.

Carlyle, J.R., Michie, A.M., Furlonger, C., Nakano, T., Lenardo, M.J., Paige, C.J., and Zuniga-Pflucker, J.C. (1997). Identification of a novel developmental stage marking lineage commitment of progenitor thymocytes. J. Exp. Med. *186*, 173–182.

Chen, H., Charlat, O., Tartaglia, L.A., Woolf, E.A., Weng, X., Ellis, S.J., Lakey, N.D., Culpepper, J., Moore, K.J., Breitbart, R.E., et al. (1996). Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. Cell *84*, 491–495.

Cioffi, J.A., Shafer, A.W., Zupancic, T.J., Smith-Gbur, J., Mikhail, A., Platika, D., and Snodgrass, H.R. (1996). Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. Nat. Med. *2*, 585–589.

Cumano, A., Paige, C., Iscove, N.N., and Brady, G. (1992). Bipotential precursor of B cells and macrophages in murine fetal liver. Nature *356*, 612–615.

Davidson, W., Pierce, J., Rudikoff, S., and Morse, H. (1988). Relationship between B cell and myeloid differentiation. Studies with a B lymphocyte progenitor line, HAFTL-1. J. Exp. Med. *168*, 389–407.

Gainsford, T., Willson, T.A., Metcalf, D., Handman, E., McFarlane, C.A., Nicola, N.A., Alexander, W.S., and Hilton, D.J. (1996). Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. Proc. Natl. Acad. Sci. USA *93*, 14564–14568.

Galy, A., Travis, M., Cen, D., and Chen, B. (1995). Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. Immunity *3*, 459–473.

Georgopoulos, K., Bigby, M., Wang, J.H., Molnar, A., Wu, P., Winandy, S., and Sharpe, A. (1994). The Ikaros gene is required for the development of all lymphoid lineages. Cell *79*, 143–156.

Godfrey, D.I., and Zlotnik, A. (1993). Control points in early T-cell development. Immunol. Today *14*, 547–553.

Hattori, N., Kawamoto, H., and Katsura, Y. (1996). Isolation of the most immature population of murine fetal thymocytes that includes progenitors capable of generating T, B, and myeloid cells. J. Exp. Med. *184*, 1901–1908.

Hirayama, F., and Ogawa, M. (1994). Cytokine regulation of early B-lymphopoiesis assessed in culture. Blood Cells *20*, 341–347.

Karasuyama, H., and Melchers, F. (1988). Establishment of mouse cell lines which constitutively secrete large quantitites of interleukin 2, 3, 4, or 5 using modified cDNA expression vectors. Eur. J. Immunol. *18*, 97–104.

Kawamoto, H., Ohmura, K., and Katsura, Y. (1997). Direct evidence for the commitment of hematopoietic stem cells to T, B and myeloid lineages in murine fetal liver. Int. Immunol. *9*, 1011–1019.

Kee, B.L., Cumano, A., Iscove, N.N., and Paige, C.J. (1994). Stromal cell independent growth of bipotent B cell-macrophage precursors from murine fetal liver. Int. Immunol. *6*, 401–407.

Keller, G. (1992). Hematopoietic stem cells. Curr. Opin. Immunol. *4*, 133–139.

Klinken, P., Alexander, W., and Adams, J. (1988). Hemopoietic lineage switch: *v-raf* oncogene converts Eμ-*myc* transgenic B cells into macrophages. Cell *53*, 857–867.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661–672.

Lanier, L.L., Spits, H., and Phillips, J.H. (1992). The developmental relationship between NK cells and T cells. Immunol. Today *13*, 392–395.

Lee, G.H., Proenca, R., Montez, J.M., Carroll, K.M., Darvishzadeh, J.G., Lee, J.I., and Friedman, J.M. (1996). Abnormal splicing of the leptin receptor in diabetic mice. Nature *379*, 632–635.

Matsuzaki, Y., Gyotoku, J.I., Ogawa, M., Nishikawa, S.I., Katsura, Y., Gachelin, G., and Nakauchi, H. (1993). Characterization of *c-kit* positive intrathymic stem cells that are restricted to lymphoid differentiation. J. Exp. Med. *178*, 1283–1292.

Metcalf, D. (1977). Hemopoietic colonies: *in vitro* cloning of normal and leukemic cells. Recent Results Cancer Res. *61*, 1–227.

Mikhail, A.A., Beck, E.X., Shafer, A., Barut, B., Gbur, J.S., Zupancic, T.J., Schweitzer, A.C., Cioffi, J.A., Lacaud, G., Ouyang, B., et al. (1997). Leptin stimulates fetal and adult erythroid and myeloid development. Blood *89*, 1507–1512.

Moore, T.A., and Zlotnik, A. (1995). T-cell lineage commitment and cytokine responses of thymic progenitors. Blood *86*, 1850–1860.

Morrison, S.J., Uchida, N., and Weissman, I.L. (1995). The biology of hematopoietic stem cells. Annu. Rev. Cell Dev. Biol. *11*, 35–71.

Orkin, S. (1992). GATA-binding transcription factors in hematopoietic cells. Blood *80*, 575–581.

Peault, B., Khazaal, I., and Weissman, I.L. (1994). *In vitro* development of B cells and macrophages from early mouse fetal thymus. Eur. J. Immunol. *24*, 781–784.

Principato, M., Cleveland, J.L., Rapp, U.R., Holmes, K.L., Pierce, J.H., Morse, H.C., and Klinken, S.P. (1990). Transformation of murine bone marrow cells with combined v-raf-v-myc oncogenes yields clonally related mature B cells and macrophages. Mol. Cell Biol. *10*, 3562–3568.

Rodewald, H.R., Moingeon, P., Lucich, J., Dosiou, C., Lopez, P., and Reinherz, E. (1992). A population of early fetal thymocytes expressing FcγRII/III contains precursors of T lymphocytes and natural killer cells. Cell *69*, 139–150.

Rolink, A., Kudo, A., Karasuyami, H., Kikushi, Y., and Melchers, F. (1991). Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells *in vitro* and *in vivo*. EMBO J. *10*, 327–336.

Sanchez, M.J., Muench, M.O., Roncarolo, M.G., Lanier, L.L., and Phillips, J.H. (1994). Identification of a common T/natural killer cell progenitor in human fetal thymus. J. Exp. Med. *180*, 569–576.

Takai, T., Li, M., Sylvestre, D., Clynes, R., and Ravetch, J. (1994). FcR γ chain deletion results in pleiotropic effector cell defects. Cell *76*, 519–529.

Wu, L., Antica, M., Johnson, G., Scollay, R., and Shortman, K. (1991). Developmental potential of the earliest precursor cells from the adult mouse thymus. J. Exp. Med. *174*, 1617–1627.

Wu, L., Li, C.L., and Shortman, K. (1996). Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. J. Exp. Med. *184*, 903–911.

Wu, L., Nichogiannopoulou, A., Shortman, K., and Georgopoulos, K. (1997). Cell-autonomous defects in dendritic cell populations of Ikaros mutant mice point to a developmental relationship with the lymphoid lineage. Immunity *7*, 483–492.