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Identification of pro-thymocytes in murine fetal blood: T lineage commitment can precede thymus colonization

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Phenotype and commitment of thymus-colonizing precursors are unknown. Here we report the identification of T lineage-committed precursors (designated pro-thymocytes) in murine fetal blood at day 15.5 of development. Fetal blood pro-thymocytes are Thy-1$^+$c-kit^{low}CD3$^-$ in contrast to fetal blood-derived pluripotent hematopoietic progenitors which are Thy-1$^-$c-kit$^+$. Upon transfer into the thymus, fetal blood pro-thymocytes generate a single wave of CD4$^+$CD8$^+$ thymocytes and subsequently mature TCR$\alpha\beta^+$ peripheral T cells. However, fetal blood pro-thymocytes lack multipotent progenitor potential since they fail to reconstitute B lymphocytes and myeloid and erythroid lineages. In contrast, T and B lymphocytes as well as myeloid and erythroid lineages are reconstituted from fetal blood-derived pluripotent progenitors. Pro-thymocytes are equally present in peripheral blood of athymic fetal mice, suggesting that this novel precursor population is T lineage-committed prior to thymus colonization and represents the earliest T lineage precursor identified.

Key words: fetal blood/hematopoiesis/pro-thymocytes/T lineage commitment/thymus colonization

Introduction

In fetal life, the colonization of the thymus by progenitor cells begins around day 11 of mouse gestation (Auerbach, 1961; Owen and Ritter, 1969). Intrathymic developmental stages have been characterized in detail by the analysis of cell surface phenotype, T cell receptor (TCR) gene rearrangements and developmental kinetics. Recently, the analysis of developmental arrests or deviations due to mutations introduced into the germline of mice has revealed the role of single gene products at distinct stages of thymocyte development (for a review see Pfeffer and Mak, 1993). Thymocytes rearrange their TCR genes at the CD4$^-$CD8$^-$ (DN) stage, and pass through a CD3$^-$CD8^{low} stage into the CD4$^+$CD8$^+$ (DP) TCR$^{-/low}$ stage. Based on their TCR specificity, DP thymocytes are selected into CD4$^+$ or CD8$^+$ TCR^{high} single positive (SP) mature thymocytes which populate the peripheral lymphoid organs (for reviews see Adkins *et al.*, 1987; Scollay *et al.*, 1988; Fowlkes and Pardoll, 1989; von Boehmer, 1990; Nikolic-Zugic, 1991; Kruisbeek, 1993).

The development of hematopoietic organs, including the thymus, is dependent on an inflow of blood-borne stem cells (Moore and Owen, 1967a,b; Metcalf and Moore, 1971; Le Douarin, 1984). The concept that fetal blood is rich in hematopoietic progenitors has been confirmed both experimentally and clinically. Between days 10 and 13 in the developing mouse, precursors for *in vivo* colony-forming cells (CFC) (Moore and Metcalf, 1970) and for pre-B cells (Melchers and Abramczuk, 1980) were demonstrated in the circulation. Human fetal and neonatal blood was shown to contain precursors for erythroid and myeloid lineages at high levels (Linch *et al.*, 1982; Nakahata and Ogawa, 1982; Leary *et al.*, 1984; Migliaccio *et al.*, 1986). Moreover, total cord blood has been used to substitute for bone marrow as a source of transplantable hematopoietic stem cells in human (Gluckman *et al.*, 1989). Nevertheless, information on the phenotypes and developmental potential of circulating progenitor cells is limited. Specifically, it has remained an open question whether thymus-colonizing cells contain T lineage-restricted prethymic progenitors or represent exclusively multilineage precursors which are induced to develop along the T lineage pathway only after entry into the thymic microenvironment (for review see O'Neill, 1991; Ikuta *et al.*, 1992).

Reconstitution of thymopoiesis in irradiated recipient mice from bone marrow has been analyzed in detail (Kadish and Basch, 1976). Within bone marrow, a hematopoietic stem cell (HSC) population characterized as Thy-1^{low}Sca-1$^+$ lineage marker negative (Lin$^-$) has been identified which reconstitutes all hematopoietic lineages, including T cells, in lethally irradiated mice (Spangrude *et al.*, 1988a,b). This demonstration implies, but does not prove, that HSC are capable of direct thymic reconstitution, without primary seeding into the bone marrow, and subsequent secondary colonization of the thymus. Evidence for the existence of T lineage-restricted bone marrow precursors has been reported from transplantation experiments using radiation-induced (Abramson *et al.*, 1977) and retroviral (Keller *et al.*, 1985) chromosome markers. Since total bone marrow cells repopulate the thymus in lethally irradiated mice within 4 h after intravenous injection (Spangrude and Weissman, 1988), it is possible that bone marrow contains T lineage-committed progenitors distinct from HSC, and that under physiological conditions putative pro-T cells dominate thymopoesis. However, no intermediate precursors between HSC and the earliest thymocytes have been identified until now.

The earliest progenitors inside the thymus were reported to express progenitor activity for both the TCR$\alpha\beta$ lineage (for a review see Fowlkes and Pardoll, 1989) and TCR$\gamma\delta$ lineage (for a review see Raulet, 1989). In addition, the

earliest thymocytes were shown to contain precursors with developmental potential for B lymphocytes (Wu *et al.*, 1991a; Matsuzaki *et al.*, 1993), natural killer cells (Rodewald *et al.*, 1992; Matsuzaki *et al.*, 1993) and, at very low frequencies, thymic dendritic cells (Ardavin *et al.*, 1993). These findings suggest that at least a subpopulation of thymus-colonizing cells is not exclusively T lineage-restricted. However, early intrathymic progenitors express myeloid precursor potential at very low frequencies (approximately one CFU-S_{d12} per 2×10^5 $CD3^-CD4^-CD8^-$ thymocytes) (Papiernik *et al.*, 1988; Wu *et al.*, 1991a; Matsuzaki *et al.*, 1993), while multipotent stem cells are highly enriched (approximately one CFU-S_{d12} per 10 HSC) for myeloid precursors (Spangrude *et al.*, 1988a). Thus, HSC activity cannot be recovered readily from fetal or adult thymus.

To address the question of whether T lineage commitment can precede thymus colonization, we began to search for hematopoietic progenitor populations in peripheral blood of fetal mice, reasoning that thymus-colonizing precursors, including but perhaps not restricted to HSC, should be present in the circulation prior to their entry into the thymus.

By phenotypic analysis of the leukocyte fraction in fetal blood at day 15.5 of gestation (FBd15.5) for expression of Thy-1 versus c-kit, we have identified two distinct hematopoietic precursor populations. In this report we characterize one multipotent fetal blood population (FB $Thy\text{-}1^-c\text{-}kit^+$) which reconstitutes both T and B lymphocyte lineages *in vivo*. Moreover, the same fetal blood population also contains precursors (CFC) which form myeloid and erythroid colonies at high frequencies *in vitro*. In addition to this multipotent FB $Thy\text{-}1^-c\text{-}kit^+$ population, we describe phenotype and developmental potential of a novel fetal blood-derived population defined as $Thy\text{-}1^+c\text{-}kit^{low}CD3^-$. Interestingly, FB $Thy\text{-}1^+c\text{-}kit^{low}CD3^-$ cells exclusively reconstitute the T cell lineage *in vivo*, but lack detectable precursor potential for B lymphocytes and myeloid and erythroid lineages. The presence of the corresponding cell population (FBd15.5 $Thy\text{-}1^+CD3^-c\text{-}kit^{low}$) in blood from athymic C57Bl/6 (nu/nu) fetal mice at day 15.5 of gestation, suggests that this population represents a prethymic stage of T lineage development. These experiments strongly support a model of thymus colonization by a novel pro-thymocyte population which is T lineage-committed prior to thymus colonization.

Results

Phenotypic analysis of fetal blood leukocytes at day 15.5 of gestation for expression of Thy-1 versus c-kit identifies several distinct subpopulations

Since the generation of T cells in the thymus depends on the continuous seeding of precursors into the thymus and since these precursors have to be transported from the primary sites of fetal hematopoiesis (initially the yolk sac and subsequently the fetal liver) to the thymus via the circulation, we focused our experiments to identify candidate thymus-colonizing cell populations on the leukocyte fraction in fetal blood. The search in fetal blood at day 15.5 of gestation, but not adult blood, offers the advantage that the former contains neither mature αβ T cells nor B lymphocytes, which together constitute ~75% of all leukocytes in adult murine blood. In particular, at day 15.5 of gestation, αβ T cell development has not progressed beyond the immature $CD4^-CD8^-$ (DN) stage in the thymus.

T lineage cells can be characterized by expression of the cell surface glycoprotein Thy-1. While Thy-1 expression is high on thymocytes, low levels of Thy-1 were reported on Sca-1^+Lin^- HSC (Spangrude *et al.*, 1988a), as well as on the surface of immature $B220^{low}$ pro-B cells (for a review see Rolink and Melchers, 1991). To determine the proportion of $Thy\text{-}1^+$ cells among the $CD45^+$ fraction in fetal blood, fetal blood leukocytes ($\rho \geq 1.056$ g/ml; see Materials and methods) were tested for expression of CD45 versus Thy-1 by flow cytometry. This analysis revealed that a major proportion (~30%) of $CD45^+$ cells in fetal blood express Thy-1 at low to intermediate levels (Figure 1A).

Since HSC, as well as several immature but lineage-committed precursors, express the receptor-type tyrosine kinase c-kit (Ogawa *et al.*, 1991; Ikuta and Weissman, 1992; for a review see Galli *et al.*, 1994), we next analyzed FB15.5 cells for expression of Thy-1 versus c-kit. Interestingly, this analysis identified four different subpopulations (Figure 1B): $Thy\text{-}1^+c\text{-}kit^{-/low}$, $Thy\text{-}1^-c\text{-}kit^+$, $Thy\text{-}1^{low}c\text{-}kit^{high}$ and $Thy\text{-}1^-c\text{-}kit^-$.

To determine whether the $Thy\text{-}1^+c\text{-}kit^{-/low}$ fraction contains mature, extrathymically derived, TCR^+ T cells, FB $Thy\text{-}1^+c\text{-}kit^{-/low}$ and FB $Thy\text{-}1^-c\text{-}kit^+$ were isolated by cell sorting and restained for CD3 expression. Consistent with the idea that FB $Thy\text{-}1^-c\text{-}kit^+$ cells are very immature, uncommitted progenitors, this population is $CD3^-$ (Figure 1E). Surprisingly, however, a large fraction (~70%) of FB $Thy\text{-}1^+c\text{-}kit^{-/low}$ expresses CD3 on the cell surface (Figure 1D). To test whether this population in fetal blood belongs to the γδ T cell lineage, sorted $Thy\text{-}1^+CD3^+$ fetal blood lymphocytes were analyzed with an anti-pan γδ TCR antibody (3A10) (Itohara *et al.*, 1989). The $Thy\text{-}1^+CD3^+$ population contains γδ TCR lineage cells since this population reacts with mAb 3A10 (data not shown). These γδ TCR lineage cells present in fetal blood at day 15.5 are extrathymically derived since this population is equally present in fetal blood of athymic C57Bl/6 nu/nu mice (data not shown). Thus, the FB $Thy\text{-}1^+c\text{-}kit^{-/low}$ fraction can be subdivided into a $CD3^+$ and a $CD3^-$ population (Figure 1B and D).

Sorted subpopulations were re-analyzed for c-kit expression (Figure 1F–H), forward/side scatter (Figure 1I–K) and morphology following Giemsa staining (Figure 1L and M). The FB $Thy\text{-}1^+CD3^-$ population expresses low levels of c-kit (Figure 1F) when compared with FB $Thy\text{-}1^-c\text{-}kit^+$ cells (Figure 1H). The FB $Thy\text{-}1^+c\text{-}kit^{low}CD3^-$ population will subsequently be denoted as FB $Thy\text{-}1^+c\text{-}kit^{low}$. Both FB $Thy\text{-}1^+c\text{-}kit^{low}$ and FB $Thy\text{-}1^-c\text{-}kit^+$ cells represent homogeneous cell populations by forward/side scatter analysis (Figure 1I and K), and display weakly (FB $Thy\text{-}1^+c\text{-}kit^{low}$; Figure 1L) or strongly (FB $Thy\text{-}1^-c\text{-}kit^+$; Figure 1M) basophilic cytoplasm and large nucleus to cytoplasm ratios. In contrast, $Thy\text{-}1^+CD3^+$ T cells are $c\text{-}kit^-$ (Figure 1G) and more heterogeneous by forward/side scatter (Figure 1J) and morphology (data not shown).

All of these FB subpopulations are generated independently from the thymus since each population is present in

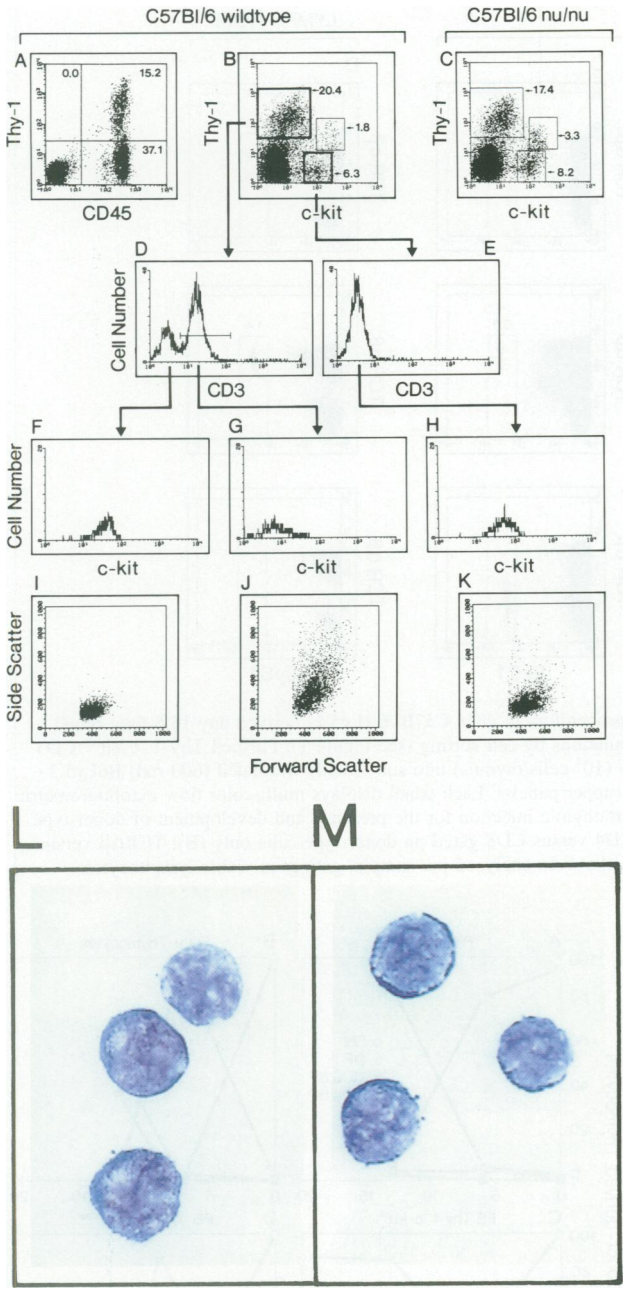

Fig. 1. Phenotypic characterization of fetal blood leukocytes from day 15.5 of gestation and purification of fetal blood hematopoietic progenitors. The leukocyte fraction in fetal blood derived from timed-pregnant (day 15.5 of gestation) C57Bl/6 mice (**A** and **B**) or athymic C57/Bl/6 (nu/nu) mice (**C**) was enriched by density gradient centrifugation ($\rho \geq 1.056$ g/ml) and analyzed by flow cytometry for expression of Thy-1 versus CD45 (A) and Thy-1 versus c-kit (B and C). Numbers in the individual quadrants and gates indicate percentages. Fetal blood leukocytes were further purified by FACS into Thy-1$^+$c-kit$^{-/low}$ and Thy-1$^-$c-kit$^+$ subpopulations according to the gates shown (B) and subsequently restained for CD3 expression (**D** and **E**). The histogram region shown in (D) contains 70% CD3$^+$ cells all of which are TCR$\gamma\delta^+$ (data not shown). The levels of c-kit expression are shown on sorted Thy-1$^+$CD3$^-$ (**F**), Thy-1$^+$CD3$^+$ (**G**) and Thy-1$^-$c-kit$^+$ (**H**) subpopulations. Forward/side scatter analysis is shown for Thy-1$^+$c-kit^{low}CD3$^-$ (**I**), Thy-1$^+$c-kit$^-$CD3$^+$ (**J**) and Thy-1$^-$c-kit$^+$ (**K**), and morphology (×650) of Giemsa-stained cytospin preparations of purified Thy-1$^+$c-kit^{low}CD3$^-$ and Thy-1$^-$c-kit$^+$ is shown in (**L**) and (**M**), respectively. Subpopulations were at least 99% pure on re-analysis.

both fetal blood derived from euthymic C57Bl/6 (Figure 1B) mice, as well as from athymic C57Bl/6 (nu/nu) mice (Figure 1C) at day 15.5 of gestation. Although not shown, the phenotype of C57Bl/6 (nu/nu)-derived fetal blood subpopulations is identical to that displayed in Figure 1D–K for fetal blood from euthymic mice.

Fetal blood at day 15.5 of gestation contains ~2000 CD45$^+$ cells/μl of total blood, which is approximately five times less than adult blood. Thus, ~600 Thy-1$^+$ cells/μl blood are present at day 15.5 of gestation. Of all Thy-1$^+$ cells, ~90% are c-kit$^{-/low}$ and ~10% are c-kit^{high} (Figure 1B and C). In addition, only ~30% of all Thy-1$^+$c-kit$^{-/low}$ are CD3$^-$ (Figure 1D). Thus, day 15.5 fetal blood contains ~160 Thy-1$^+$c-kit^{low}CD3$^-$ cells/μl blood. If we assume that 8% of the body weight constitutes blood, and take the body weight of the fetal mouse at day 15.5 of gestation as 350 mg, one can estimate the total number of Thy-1$^+$c-kit^{low}CD3$^-$ cells to be ~4500/fetus.

Both FB Thy-1$^+$ c-kit^{low} and FB Thy-1$^-$ c-kit$^+$ contain αβ T lineage precursors in vivo

To analyze fetal blood subpopulations for their developmental potential to generate T cells *in vivo*, day 15.5 FB Thy-1$^-$c-kit$^+$ and FB Thy-1$^+$c-kit^{low} derived from C57Bl/6 mice (Ly5.1$^+$) were enriched by density gradient centrifugation and subsequently isolated by fluorescence-activated cell sorting (FACS). Each population was found to be >99% pure. Subsequently, from each population 10^4 cells were injected intrathymically into 600 rad irradiated congenic B6Ly5.2 recipient mice (Ly5.2$^+$). Thirteen days post-injection, thymus and lymph nodes of recipient mice were analyzed by three-color immunofluorescence analysis for expression of the donor marker (Ly5.1) as well as CD4 and CD8, to determine the presence and developmental progression of donor-type cells. Uninjected control mice contain, as expected, no donor-derived thymocytes (Figure 2, top row). In contrast, Ly5.1$^+$ thymocytes are present in both recipient mice receiving either FB Thy-1$^+$c-kit^{low} (Figure 2, second row) or FB Thy-1$^-$c-kit$^+$ (Figure 2, third row). After gating on donor-derived thymocytes (Figure 2A), it is apparent that 13 days after cell transfer most thymocytes derived from the FB Thy-1$^-$c-kit$^+$ population are at the immature CD4$^+$CD8$^+$ DP stage (88.4%), while only few are CD4 or CD8 SP cells (3.1 and 3.7%, respectively; Figure 2B, third row). Consequently, most Ly5.1$^+$ thymocytes are TCR$^{-/low}$ at this stage of development (Figure 2C, third row).

Interestingly, the progeny of FB Thy-1$^+$c-kit^{low} also gives rise to developing thymocytes, but at this time point (13 days after cell transfer) most donor-derived cells have already progressed to the more mature CD4 or CD8 SP stage (52.7 and 32.2%, respectively), while only 11.6% are still at the DP stage (Figure 2B, second row). In agreement with this more mature stage of thymocyte differentiation, most Ly5.1$^+$ thymocytes are now TCR^{high} (Figure 2C, second row). To test whether donor-derived thymocytes also give rise to peripheral T cells, lymph nodes of recipient mice were analyzed for the presence of Ly5.1$^+$ cells. Consistent with the mature stage of intrathymic development of progeny derived from FB Thy-1$^+$c-kit^{low} precursors, Ly5.1$^+$TCRαβ$^+$ T cells are now present in lymph nodes (1.4%; Figure 2D, second

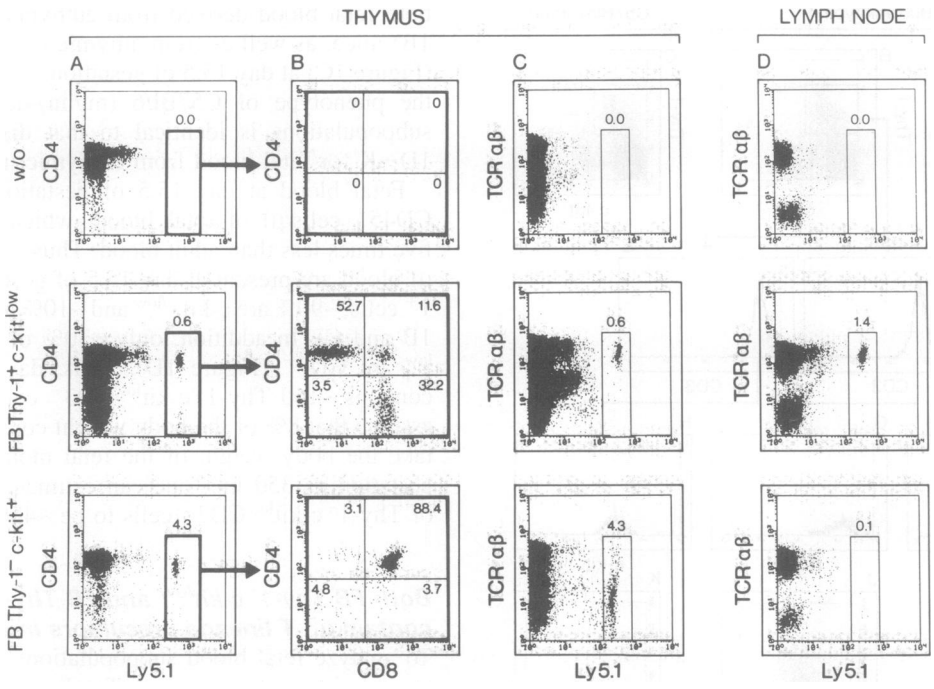

Fig. 2. Fetal blood Thy-1$^+$c-kit^{low}CD3$^-$ and Thy-1$^-$c-kit$^+$ contain αβ T cell progenitors *in vivo*. C57Bl/6 (Ly5.1)-derived day 15.5 fetal blood leukocytes were separated into Thy-1$^+$c-kit^{low}CD3$^-$ and Thy-1$^-$c-kit$^+$ subpopulations by cell sorting (see Figure 1). Purified Thy-1$^+$c-kit^{low}CD3$^-$ (middle panels) and Thy-1$^-$c-kit$^+$ (lower panels) were injected intrathymically (10^4 cells/thymus) into sub-lethally irradiated (600 rad) B6Ly5.2 congenic recipient mice. Uninjected control animals were analyzed in parallel (upper panels). Each panel displays multi-color flow cytofluorometric analysis of either thymocytes (**A**–**C**) or lymph node cells (**D**) 13 days after intrathymic injection for the presence and development of donor-type (Ly5.1$^+$) cells. Two parameters are shown at a time: CD4 versus Ly5.1 (A); CD4 versus CD8 gated on donor-type cells only (B), TCRαβ versus Ly5.1 (C and D); 5×10^4 total (A, C and D) and 5×10^3 donor-type gated (B) cells were analyzed per sample, gating on viable lymphocytes. Numbers given in the individual quadrants and gates indicate percentages.

row). In contrast, at this time after transfer the progeny of FB Thy-1$^-$c-kit$^+$ cells, which dominantly has a DP phenotype inside the thymus (Figure 2B, third row), has not yet migrated to the periphery (Figure 2D, third row).

The kinetics of intrathymic T cell development of FB Thy-1$^-$c-kit$^+$ and FB Thy-1$^+$c-kit^{low} are shown in Figure 3. For comparison, control mice were injected intrathymically with 5×10^4 total day 15.5 fetal liver cells (FL) (Figure 3A) or 5×10^4 total day 15.5 fetal thymocytes (FT) (Figure 3B). Intrathymic T cell development is delayed by ~7 days when comparing FL with FT precursors (compare Figure 3A and B). Interestingly, FB Thy-1$^-$c-kit$^+$ precursors mature intrathymically with slow, fetal liver-like kinetics (Figure 3C). In contrast, FB Thy-1$^+$c-kit^{low} display a faster development which resembles the speed of maturation of adoptively transferred fetal thymocytes (compare Figure 3B with D). All four tested precursor populations (FL, FT, FB Thy-1$^+$c-kit^{low} and FB Thy-1$^-$c-kit$^+$) give rise to mature peripheral αβ T cells (data not shown). Within ~13 days, both total FT and FB Thy-1$^+$c-kit^{low} generate a single wave of DP and subsequently SP thymocytes, indicating their limited self-renewal capacity. In contrast, 20 days post-injection of FB Thy-1$^-$c-kit$^+$ the recipient thymus still contains, in addition to mature CD4 SP (~6%) and CD8 SP (~2%) cells, ~90% DP thymocytes.

Finally, the capacity of FB Thy-1$^-$c-kit$^+$ and FB Thy-1$^+$c-kit^{low} cells to reconstitute the thymus from the bloodstream following intravenous transfer was also investigated. In recombination-activating gene-deficient (RAG$^{-/-}$) mice thymocyte development is arrested at

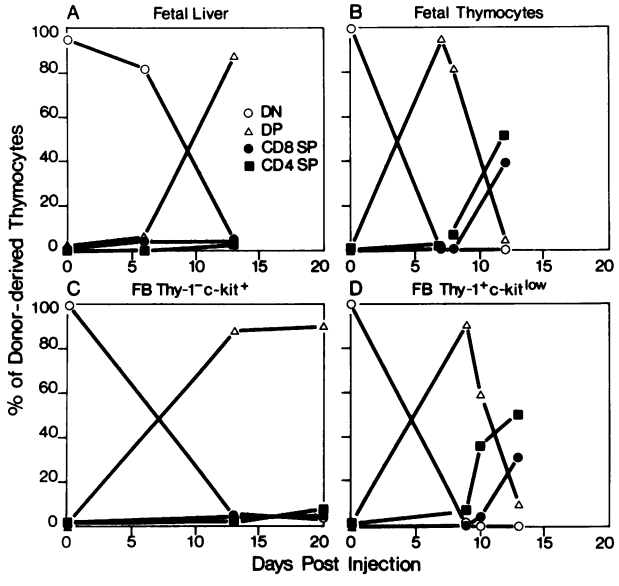

Fig. 3. Fetal blood Thy-1$^+$c-kit^{low}CD3$^-$ and Thy-1$^-$c-kit$^+$ mature intrathymically with distinct kinetics *in vivo*. The intrathymic differentiation kinetics of cellular progeny of total fetal liver cells (**A**), total fetal thymocytes (**B**), fetal blood Thy-1$^-$c-kit$^+$ (**C**) and Thy-1$^+$c-kit^{low}CD3$^-$ (**D**) subpopulations derived from day 15.5 of gestation were analyzed at the indicated time points after intrathymic injections. Thymocytes were stained simultaneously with antibodies against the donor marker (Ly5.1), CD4 and CD8, and analyzed using three-color flow cytofluorometry (5×10^4 cells/sample). Percentages were determined by gating on donor-type cells only. Cells at the zero time point represent the day of injection.

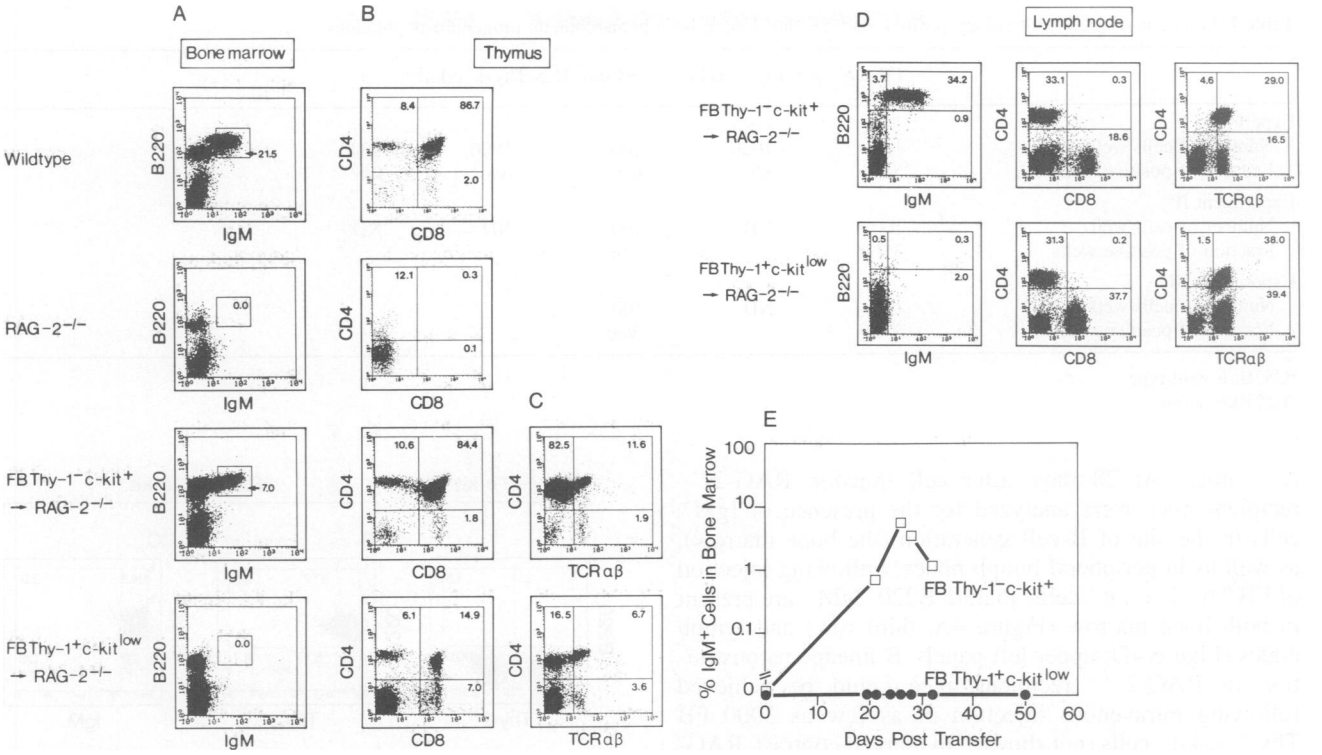

Fig. 4. Fetal blood Thy-1$^-$c-kit$^+$ and Thy-1$^+$c-kit^{low}CD3$^-$ reconstitute the T cell lineage but only Thy-1$^-$c-kit$^+$ reconstitutes the B cell lineage *in vivo* following intravenous transfer. Bone marrow cells (**A**), thymocytes (**B** and **C**) and lymph node cells (**D**) from C57Bl/6 wild-type (A and B, top row), RAG-2-deficient (A and B, second row) and 400 rad-irradiated RAG-2$^{-/-}$ recipient mice after intravenous injection of either 2.5×10^4 FB Thy-1$^-$c-kit$^+$ (A–C, third row; D, upper row) or 2.5×10^4 FB Thy-1$^+$c-kit^{low} (A–C, fourth row; D, lower row), respectively, were analyzed 28 days after cell transfer for expression of B220 versus IgM (A and D, left panels), CD4 versus CD8 (B and D, middle panels) or CD4 versus TCRαβ (C and D, right panels). Cells were stained simultaneously with antibodies against B220 and IgM, CD4 and CD8, or CD4 and TCRαβ, and analyzed, after gating on lymphoid cells, using two-color flow cytofluorometry (5×10^4 cells/sample). Numbers shown indicate percentages in the gates (A) or individual quadrants (B–D). The kinetics of B cell reconstitution *in vivo* are displayed in (**E**). RAG-2$^{-/-}$ mice received fetal blood subpopulations as indicated for (A) and recipient bone marrow was analyzed as described in (A) at indicated times after intravenous cell transfer.

the transition from the CD3$^-$CD4$^-$CD8$^-$ to the CD3$^{-/low}$CD4$^+$CD8$^+$ stage (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992; Figure 4B, second row), causing a lack of mature peripheral T cells. This developmental defect can be overcome by introducing wild-type hematopoietic progenitors in either genetically chimeric mice (Chen *et al.*, 1993) or fetal liver or bone marrow chimeras (data not shown). The use of RAG-deficient mice, but not lethally irradiated mice, offers the advantage that the former do not depend on HSC transfer for their survival since non-lymphocyte lineages are not affected by the mutation. To facilitate hematopoietic reconstitution, RAG-2$^{-/-}$ recipient mice were irradiated with 400 rad and subsequently injected intravenously with 2.5×10^4 FB Thy-1$^-$c-kit$^+$ or 2.5×10^4 FB Thy-1$^+$c-kit^{low} cells. At 28 days post-injection, recipient bone marrow, thymus and lymph nodes were analyzed for the presence of mature IgM$^+$ B cells (see below) and TCR$^+$ T cells. Consistent with the results obtained following intrathymic injections (Figures 2 and 3), both fetal blood-derived progenitor populations reconstitute thymopoiesis (Figure 4B, third and fourth row). Both CD4 SP (Figure 4C) and CD8 SP (data not shown) thymocytes are TCRαβ$^+$ and colonize lymph nodes as mature CD4$^+$ or CD8$^+$ T cells (Figure 4D, middle and right panels). These experiments provide direct evidence that both FB Thy-1$^-$c-kit$^+$ and FB Thy-1$^+$c-kit^{low} populations reconstitute the αβ T cell lineage also following intravenous transfer *in vivo*.

Collectively, these experiments demonstrate that both phenotypically distinct fetal blood-derived populations, FB Thy-1$^-$c-kit$^+$ and FB Thy-1$^+$c-kit^{low}, contain precursors for mature T cells *in vivo*. Importantly, as shown in Figures 2 and 3, FB Thy-1$^-$c-kit$^+$ and FB Thy-1$^+$c-kit^{low} represent distinct precursor populations since their progeny develop along the αβ T cell pathway with kinetics that differ by ~5 days *in vivo*, indicating that FB Thy-1$^+$c-kit^{low} are more mature than FB Thy-1$^-$c-kit$^+$ cells.

FB Thy-1$^+$c-kit^{low}, but not FB Thy-1$^-$c-kit$^+$, cells fail to reconstitute the B cell lineage in vivo and in vitro

In contrast to bone marrow derived from wild-type mice which contains B220$^+$IgM$^-$ B cell precursors as well as B220$^+$IgM$^+$ B cells (Figure 4A, top panel), B cell development in bone marrow derived from lymphocyte-deficient RAG-1$^{-/-}$ or RAG-2$^{-/-}$ mice is arrested at the immature B220$^+$IgM$^-$ stage (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992; Figure 4A, second row). In the absence of competition from endogenous lymphocyte precursors, RAG-deficient recipient mice may provide a sensitive *in vivo* system to study B lymphopoiesis from hematopoietic precursors.

To analyze the developmental potential for the generation of B lymphocytes from FB precursors *in vivo*, 2.5×10^4 day 15.5 FB Thy-1$^-$c-kit$^+$ or 2.5×10^4 FB Thy-1$^+$c-kit^{low} were injected intravenously into 400 rad irradiated RAG-

Table I. Fractions of pre-B cell colony-positive cultures developing from hematopoietic progenitor populations

	FB day 15.5 Thy-1$^-$c-kit$^+$		FB day 15.5 Thy-1$^+$c-kit^{low}		
Experiment I[a]					
Number of cells/well	100	1000	100	1000	2000
Fraction of positive wells	5/8	8/8	0/8	0/8	0/8
Experiment II[a]					
Number of cells/well	100	ND	100	ND	ND
Fraction of positive wells	35/96		0/96		
Experiment III[b]					
Number of cells/well	100	ND	100		
Fraction of positive wells	65/96		0/60		

[a]C57Bl/6 wild-type.
[b]C57Bl/6 nu/nu.

$2^{-/-}$ mice. At 28 days after cell transfer, RAG-$2^{-/-}$ recipient mice were analyzed for the presence of IgM$^+$ cells in the site of B cell generation (the bone marrow), as well as in peripheral lymph nodes. Following injection of FB Thy-1$^-$c-kit$^+$ cells, mature B220$^+$IgM$^+$ are present in both bone marrow (Figure 4A, third row) and lymph nodes (Figure 4D, upper left panel). B lineage reconstitution in RAG-$2^{-/-}$ recipient mice could be achieved following intravenous injection of as few as 3000 FB Thy-1$^-$c-kit$^+$ cells (not shown). In marked contrast, RAG-$2^{-/-}$ mice reconstituted with 2.5×10^4 day 15.5 FB Thy-1$^+$c-kit^{low} lack IgM$^+$ cells in the bone marrow (Figure 4A, fourth row) and in the periphery (Figure 4D, lower left panel). Transplantations of FB Thy-1$^+$c-kit^{low} cells into RAG-$2^{-/-}$ mice have been performed more than 10 times. In no instance could intravenous injection of this fetal blood population generate IgM$^+$ cells *in vivo*.

To exclude the possibility that FB Thy-1$^+$c-kit^{low} precursors give rise to B cells *in vivo* at time points other than those shown in Figure 4A and D, the kinetics of B cell reconstitution in RAG-$2^{-/-}$ recipient mice was analyzed up to 50 days post-injection (Figure 4E). While the pluripotent population defined as FB Thy-1$^-$c-kit$^+$ generates IgM$^+$ cells (range 0.6–7.0%) in the bone marrow beginning at 20 days after transfer, IgM$^+$ cells are detectable in neither the bone marrow (Figure 4E) nor the periphery (not shown) up to 50 days post-transfer of FB Thy-1$^+$c-kit^{low} cells. These experiments demonstrate that FB Thy-1$^+$c-kit^{low} cells lack the potential to generate B cells under these conditions *in vivo*.

The lack of *in vivo* generation of B cells from FB Thy-1$^+$c-kit^{low} cells could be the result of a low frequency with which this population, when compared with FB Thy-1$^-$c-kit$^+$, may be capable of generating B lymphocytes *in vivo*. To rigorously test this possibility, the capacity of both populations to develop into B cells was analyzed in a sensitive *in vitro* assay. Co-culture of hematopoietic precursors on a stroma cell (PA6) layer in the presence of interleukin-7 (IL-7) may, at present, be the most efficient system to allow the generation of pre-B cell colonies *in vitro* (Sudo *et al.*, 1989; Rolink *et al.*, 1993). To this end, cell sorter-purified FB Thy-1$^-$c-kit$^+$ or FB Thy-1$^+$c-kit^{low} cells were cultured on PA6 cells in the presence of IL-7. After 10 days, cultures were analyzed by microscopical inspection and flow cytometry for the presence or absence of pre-B cell colonies.

When FB Thy-1$^-$c-kit$^+$ cells were cultured on PA6 + IL-7, this precursor population generated pre-B cell colonies according to the Poisson distribution, at a frequency of ~1/200 cells (Table I). In contrast, FB Thy-1$^+$c-kit^{low} cells failed to form pre-B cell colonies *in vitro* under the same conditions (Table I). To verify this morphological determination, colonies were also characterized by cell surface phenotype. Cells were pooled from eight cultures and analyzed by flow cytometry for expression of B220 versus Thy-1. A representative experiment is shown in Figure 5. Cultures derived at high frequencies from FB Thy-1$^-$c-kit$^+$ cells were indeed dominantly (~90%) B220$^+$Thy-1$^-$ (Figure 5B). Expression of cell surface IgM by a fraction (~3%) of B220$^+$Thy-1$^-$ cells verified the B lineage assignment of this population (Figure 5C). In addition, these cultures contained a small subpopulation (~5%) of B220$^-$Thy-1$^+$ cells (Figure 5B). In contrast, FB Thy-1$^+$c-kit^{low} cells cultured on PA6 + IL-7 failed to form pre-B cell colonies *in vitro* (Table I). Interestingly, however, slowly growing cells could nevertheless be expanded *in vitro* from FB Thy-1$^+$c-kit^{low} cells. Consistent with the morphological determination as 'non-pre-B cells', progeny from FB Thy-1$^+$c-kit^{low} were dominantly B220$^-$Thy-1$^+$ cells. Importantly, B220$^+$Thy-1$^-$ cells did not develop (Figure 5A). The characterization, including the developmental potential, of B220$^-$Thy-1$^+$ cells derived from FB Thy-1$^+$c-kit^{low} *in vitro* remains to be determined.

Consistent with our analysis of B cell reconstitution

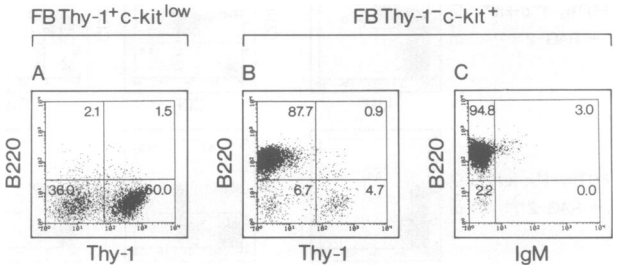

Fig. 5. Phenotype of *in vitro* colonies derived from fetal blood Thy-1$^-$c-kit$^+$ and Thy-1$^+$c-kit^{low}CD3$^-$ populations following culture on PA6 stroma cells in the presence of IL-7. Purified fetal blood Thy-1$^-$c-kit$^+$ (**B** and **C**) and Thy-1$^+$c-kit^{low} (**A**) subpopulations were cultured at 100 cells/well on PA6 stroma cells in the presence of IL-7 as described in Materials and methods. After 10 days of culture, developing colonies were pooled from eight cultures and analyzed by two-color flow cytofluorometry for expression of B220 versus Thy-1 (A and B) and B220 versus IgM (C). Numbers given in the individual quadrants indicate percentages.

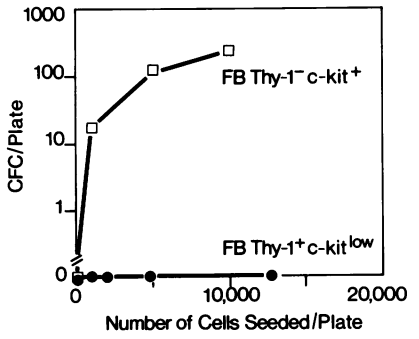

Fig. 6. Fetal blood Thy-1$^-$c-kit$^+$, but not Thy-1$^+$c-kit^{low}, cells form myeloid and erythroid colonies at high frequencies *in vitro*. Titrated numbers of purified fetal blood Thy-1$^-$c-kit$^+$ (□) or Thy-1$^+$c-kit^{low} (●) cells were cultured in 1 ml methyl cellulose medium per plate in the presence of cytokines (IL-1, IL-3, GM-CSF, SCF, Epo) to analyze their responsiveness to myeloid and erythroid growth conditions. After 10 days of culture, each plate was inspected visually to determine the number of colonies per culture. The number of colonies per input cell concentration is displayed. Colonies derived from FB Thy-1$^-$c-kit$^+$ precursors consisted of macrophages, granulocytes and erythroblasts. The data show one representative out of three independent experiments.

in vivo, we use these data to imply that the FB Thy-1$^+$c-kit^{low} population does not express the potential to generate B lymphocytes, while the FB Thy-1$^-$c-kit$^+$ population contains precursors for the B lineage at a frequency of ~1/200. The observed lack of B cell progenitor potential of FB Thy-1$^+$c-kit^{low} cells is not thymus-dependent since the same cell population derived from athymic (nu/nu) mice behaves identically (Table I, experiment III).

FB Thy-1$^+$c-kit^{low}, but not FB Thy-1$^-$c-kit$^+$, cells fail to form myeloid and erythroid colonies (CFC) in vitro

Having analyzed the precursor potential of FB Thy-1$^-$c-kit$^+$ and FB Thy-1$^+$c-kit^{low} for the generation of T and B lymphocyte lineages, respectively, both fetal blood precursor populations were next tested for their capacity to generate non-lymphoid hematopoietic lineages. For this purpose, FB Thy-1$^-$c-kit$^+$ and FB Thy-1$^+$c-kit^{low} were purified as described above and titrated into methyl cellulose-containing medium in the presence of a cocktail of growth factors [IL-1, IL-3, GM-CSF, stem cell factor (SCF) and erythropoietin] supporting myeloid and erythroid colony formation. Developing colonies were counted after 10 days of culture. In this assay, FB Thy-1$^-$c-kit$^+$ cells gave rise to myeloid colonies at a frequency of about one colony forming cell (CFC) per 100 cells plated (Figure 6). Growing colonies consisted of homogeneous as well as mixed cell types. Giemsa–May–Grünwald hematological staining of cytospin preparations of individual colonies identified macrophages, granulocytes and erythroblasts (data not shown). Thus, FB Thy-1$^-$c-kit$^+$ cells express the potential to generate myeloid and erythroid lineages. Interestingly, FB Thy-1$^+$c-kit^{low} failed to generate colonies in this assay (no CFC/12 500 cells; Figure 6). In Figure 6, representative data from one out of three independent experiments are displayed. Thus, in analogy to the results in assays measuring B cell precursor potential *in vitro*, FB Thy-1$^-$c-kit$^+$ cells are highly responsive, while the FB Thy-1$^+$c-kit^{low} population is unresponsive under these differentiation conditions *in vitro*.

Analysis of TCR β-chain gene rearrangement in fetal blood subpopulations reveals DJβ rearrangements in a fraction of FB Thy-1$^+$c-kit^{low} cells, while multipotent FB Thy-1$^-$c-kit$^+$ cells carry the DJβ locus in germline configuration

Rearrangement of B cell (Tonegawa, 1983) and T cell (Davis, 1985) antigen receptor gene loci to form functional V(D)J gene segments is one key molecular event in lymphocyte ontogeny. In αβ T cell development in the thymus, DJβ rearrangements precede V(D)J$_\beta$ rearrangements (for a review see Kronenberg *et al.*, 1986). To analyze fetal blood-derived precursor populations for their rearrangement status (DJβ versus germline configuration), genomic DNA was isolated from FB Thy-1$^-$c-kit$^+$, FB Thy-1$^+$c-kit^{low} and FB Thy-1$^+$TCRγδ$^+$ CD3$^+$ populations. Subsequently, a sensitive PCR-based method was applied utilizing sense and anti-sense primer hybridizing to sequences located 5′ of the Dβ2 and within the Jβ2.7 elements, respectively (Anderson *et al.*, 1992). This primer combination can amplify in a single PCR a germline-derived DNA fragment of 1858 bp and/or several shorter DNA products derived from DJβ rearranged loci (Dβ2Jβ2.1 to Jβ2.7 corresponding to DNA fragments ranging from 1279 to 224 bp).

When this analysis was applied in control experiments to genomic DNA from fetal liver and fetal thymus from day 15.5 of gestation, FL15.5 DNA was found to be, as expected, dominantly if not exclusively in germline configuration at the Dβ2Jβ2 locus (Figure 7, lane 1). In contrast, analysis of DNA from fetal thymocytes at day 15.5 yields, in addition to the germline band, four distinct PCR products corresponding to Dβ2Jβ2.3, Dβ2Jβ2.4, Dβ2Jβ2.5 and Dβ2Jβ2.7 rearrangements, respectively (Figure 7, lane 2). Multipotent FB Thy-1$^-$c-kit$^+$ cells and mature γδ lineage cells (FB Thy-1$^+$CD3$^+$) carry their DNA at the Dβ2 locus exclusively in germline configuration (Figure 7, lanes 3 and 5). The band of ~1100 bp present in lanes 1–5 represents a background product. Interestingly, genomic DNA from fetal blood pro-thymocytes (FB Thy-1$^+$c-kit^{low}) shows, in addition to a dominant germline band, several DJβ rearrangements (Dβ2Jβ2.3, Dβ2Jβ2.4, Dβ2Jβ2.5 and Dβ2Jβ2.7; Figure 7, lane 4). The expected PCR products hybridized with an internal oligonucleotide probe (data not shown). This result suggests that a fraction of fetal blood pro-thymocytes (FB Thy-1$^+$c-kit^{low}) initiates TCR DJβ rearrangements before thymus colonization in fetal ontogeny.

Discussion

Following the original discovery of the immunological function of the thymus (Miller, 1961; Cooper *et al.*, 1966), it was recognized, over 25 years ago, that intrathymic progenitor cells are derived from circulating ('blood-borne') stem cells (Moore and Owen, 1967a,b). Moore and Owen proposed that circulating progenitor cells develop into particular hematopoietic cell types under the influence of 'inductive microenvironments' (i.e. thymus or bursa of Fabricius in birds). This supply of blood-borne precursors colonizing the thymus is maintained throughout post-natal life (Donskoy and Goldschneider, 1992). However, the nature of thymus-colonizing progenitors has remained elusive. Consequently, the questions of whether

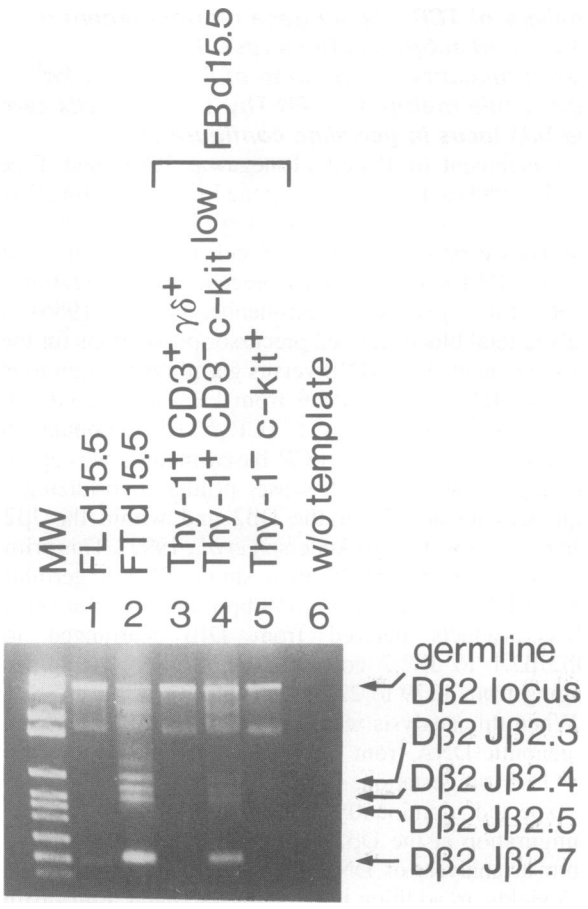

Fig. 7. A fraction of fetal blood pro-thymocytes defined as Thy-1$^+$c-kit^{low}CD3$^-$ is TCR β-chain DJ rearranged while both the multipotent fetal blood Thy-1$^-$c-kit$^+$ and Thy-1$^+$c-kit$^-$ CD3$^+$ γδ T cells carry the TCR β-chain locus in germline configuration. Genomic DNA was isolated from fetal liver cells (lane 1), fetal thymocytes (lane 2) and fetal blood subpopulations (Thy-1$^+$c-kit$^-$CD3$^+$ γδ T cells, lane 3; Thy-1$^+$c-kit^{low}CD3$^-$, lane 4; Thy-1$^-$c-kit$^+$, lane 5) obtained from fetal mice at day 15.5 of gestation. Subsequently, for each cell population TCR β-chain DJ rearrangements were analyzed by PCR (Anderson *et al.*, 1992) using amplimers recognizing sequences 5′ of the Dβ2.1 element and 3′ of the Jβ2.7 element, respectively. The distance between both PCR primers is 1858 bp in germline, and depending on the rearrangement of Dβ2.1 to either Jβ2.1, Jβ2.2, Jβ2.3, Jβ2.4, Jβ2.5, Jβ2.6 or Jβ2.7, PCR products varying in size from 1279 to 224 bp are amplified. When this analysis is applied to genomic DNA from thymocytes or lymph node cells, the preferentially amplified products correspond to either germline or Dβ2.1Jβ2.3, Dβ2.1Jβ2.4, Dβ2.1Jβ2.5 and Dβ2.1Jβ2.7 rearrangements (not shown). Fetal liver and fetal thymocytes from day 15.5 serve as negative and positive controls, respectively. The majority of the genomic DNA from fetal blood pro-thymocytes carries the TCR β-chain locus in germline configuration, but DJβ rearrangements are present in this population (lane 4). In contrast, no DJβ rearrangements are detectable in either Thy-1$^+$c-kit$^-$CD3$^+$ γδ T cells (lane 3) or the multipotent Thy-1$^-$c-kit$^+$ population (lane 5). Omission of template DNA in the reaction mixture gave no PCR product (lane 6). The band of ~1100 bp present in lanes 1–5 represents a background product. Amplified PCR products hybridized with an internal oligonucleotide probe independent from the amplimers used for PCR (not shown). Molecular weight markers shown on the left correspond to bp 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234 and 154.

T lineage commitment is exclusively occurring inside the thymic microenvironment, or whether pro-thymocytes exist which, by definition, are T lineage-committed prior to their entry into the thymus, or whether both pathways exist, have remained open.

Fetal liver and bone marrow, as the major sites of hematopoiesis, represent complex hematopoietic organs which are dominated by erythropoiesis, thrombopoiesis, myelopoiesis and B lymphopoiesis. In contrast, hematopoietic progenitor populations in fetal blood may be expected to be less complex compared with fetal liver or bone marrow. This reduced complexity has allowed us to purify and characterize from fetal blood two phenotypically and functionally distinct novel precursor populations. The first population, defined phenotypically as Thy-1$^+$c-kit^{low}CD3$^-$, fulfils the criteria of a T lineage-restricted prethymic progenitor population. The second fetal blood population, defined phenotypically as Thy-1$^-$c-kit$^+$, contains multipotent progenitors. The implications from the identification of two distinct circulating progenitor populations will be discussed separately.

Pro-thymocytes

Pro-thymocytes should fulfil the following criteria. (i) They should be defined by a unique cell surface phenotype to allow the purification of a relatively homogeneous population. (ii) Following *in vivo* transfer via intrathymic and intravenous injections they should efficiently repopulate the T cell lineage, while they should lack multilineage stem cell activity (O'Neill, 1991; Ikuta *et al.*, 1992). (iii) They should be present in a prethymic compartment (i.e. fetal liver, fetal blood or bone marrow) and consequently they should be found in euthymic as well as athymic mice. The FB Thy-1$^+$c-kit^{low}CD3$^-$ cell population satisfies these criteria.

Evidence that fetal blood pro-thymocytes represent a homogeneous population comes from the following findings. Forward/side scatter analysis (Figure 1I) and the morphological appearance of cytospin preparations (Figure 1L) demonstrate no heterogeneity of FB Thy-1$^+$c-kit^{low}CD3$^-$ cells. Moreover, further phenotypic analysis of sorted pro-thymocytes for cell surface expression of several markers [HSA, CD44 (pgp1), IL-2 receptor α-chain, TER 119 and IL-7 receptor (IL-7R); data not shown) fails to identify subsets within the pro-thymocyte population. Interestingly, all fetal blood pro-thymocytes are IL-7R$^+$ (unpublished observation) which, in conjunction with the expression of intermediate levels of Thy-1, strongly supports the notion of lymphoid commitment.

In contrast, the analysis of TCR DJβ rearrangements of fetal blood pro-thymocytes has revealed heterogeneity within FB Thy-1$^+$c-kit^{low}CD3$^-$ cells. Consistent with previous rearrangement analysis of the TCRβ locus in very early intrathymic progenitors (Trowbridge *et al.*, 1985; Wu *et al.*, 1991b), most pro-thymocytes carry their DNA in germline configuration (Figure 7, lane 4). However, several TCR DJβ rearrangements are detectable by PCR in a fraction of fetal blood pro-thymocytes (Figure 7, lane 4), but not in multipotent FB Thy-1$^-$c-kit$^+$ cells (Figure 7, lane 5). If these TCR DJβ rearrangements were derived from contaminating maternal T cells, one should find an enrichment of rearranged DNA in the FB Thy-1$^+$c-kit$^-$CD3$^+$ population. This is not the case (Figure 7, lane 3). The presence of TCR DJβ rearrangements in pro-thymocytes suggests that activation of the recombinase machinery in developing αβ T cells may not be restricted exclusively to progenitors inside the thymus. One possible explanation for the previous failure to identify TCR DJβ

rearrangements in the earliest thymocytes is the lower sensitivity of the analysis (loss of germline bands) by Southern blotting when compared with the positive identification of rearrangements by PCR used here. The presence of TCR DJβ rearrangements in a fraction of early fetal blood is reminiscent of the situation described for chicken B cell progenitors which have performed immunoglobulin D to J_H rearrangements before seeding of the bursa (Reynaud *et al.*, 1992). Collectively, the presence of TCR DJβ rearrangements detected by PCR in a fraction of fetal blood pro-thymocytes provides additional evidence for T lineage commitment of FB Thy-1$^+$c-kit^{low}CD3$^-$ cells.

Fetal blood pro-thymocytes display strong T lineage-repopulating activity *in vivo*. Intrathymic transfer of 10^4 purified FB Thy-1$^+$c-kit^{low}CD3$^-$ cells leads to a substantial contribution (1.4%) of donor-type T cells to the peripheral T cell pool (Figure 2D). If one considers the total number of peripheral T cells in the mouse to be ~10^8, then 10^4 pro-thymocytes have generated ~1.4×10^6 mature αβ T cells in 13 days. The fact that T cell development from pro-thymocytes is accelerated by ~5 days when compared with multipotent FB Thy-1$^-$c-kit$^+$ cells (Figure 3) further supports the notion that FB Thy-1$^+$c-kit^{low}CD3$^-$ cells are already T lineage-committed at the time they enter the thymus.

FB Thy-1$^+$c-kit^{low}CD3$^-$ cells are also clearly capable of thymus seeding from the bloodstream (Figure 4). Of eight recipient mice receiving fetal blood pro-thymocytes intravenously, two showed reconstitution of thymopoiesis. Detectable seeding of progenitors into the host thymus in a fraction of, but not all, recipient animals from only ~2×10^4 T lineage-committed progenitors after intravenous transfer is not surprising in view of the fact that the efficiency for thymic seeding of fetal intrathymic progenitors has been estimated to be ~500-fold lower following intravenous versus intrathymic cell transfer (Rodewald *et al.*, 1992). For a comparison of the thymic seeding capacities of various progenitor populations, it should be noted that ~5×10^5 adult (Fowlkes *et al.*, 1985) or ~2×10^6 fetal (data not shown) CD3$^-$CD4$^-$CD8$^-$ intrathymic progenitors are required to detect donor-type thymopoiesis following intravenous injection into irradiated recipient mice. Moreover, fetal blood pro-thymocytes express a limited self-renewal capacity *in vivo* as demonstrated by the transient nature of thymopoiesis following intrathymic (Figures 2 and 3) and intravenous (Figure 4) transfers. Such a lack of extensive self-renewal might be expected from a lineage-committed progenitor cell and is reminiscent of the developmental potential of DN thymocytes (Fowlkes *et al.*, 1985). In contrast, 2×10^4 multipotent FB Thy-1$^-$c-kit$^+$ cells generate sustained hematopoiesis, including thymopoiesis, for up to 6 months following intravenous transfer (data not shown), revealing engraftment and long-term reconstitution from this population.

Importantly, fetal blood pro-thymocytes are not a subset of multipotent stem cells since they lack detectable developmental potential for B cells and myeloid and erythroid cells (Figures 4–6 and Table I), even in sensitive *in vitro* assays. Thus, FB Thy-1$^+$c-kit^{low}CD3$^-$ cells represent the first prethymic cell population identified which efficiently repopulates αβ T cells while clearly lacking multipotency. Finally, the generation of fetal blood pro-thymocytes is not thymus-dependent because the same population is present in fetal blood at day 15.5 of gestation in athymic (nu/nu) mice.

Multipotent fetal blood progenitors

FB Thy-1$^-$c-kit$^+$ cells are multipotent by the following criteria. (i) FB Thy-1$^-$c-kit$^+$ cells generate thymocytes and subsequently peripheral T cells (Figure 2). Comparison of the kinetics of intrathymic reconstitution from FB Thy-1$^-$c-kit$^+$ cells with the kinetics from either total fetal liver cells (Figure 2A compared with C) or pro-thymocytes (Figure 3C compared with D) suggests that FB Thy-1$^-$c-kit$^+$ cells represent very immature precursors which first have to mature or commit themselves into a pro-thymocyte stage before they can develop further from the DN to the DP stage. Consistent with this view, we do not detect DJβ rearrangements in this population (Figure 7, lane 5). (ii) FB Thy-1$^-$c-kit$^+$ cells reconstitute the second major lymphocyte lineage, the B cell lineage, both *in vivo* (Figure 4) and at frequencies of ~1/200 *in vitro* (Table I and Figure 5B). Similar frequencies were obtained by Rolink *et al.* (1993) who analyzed c-kit$^+$B220$^-$ cells from young adult bone marrow in this assay. (iii) Under myeloid and erythroid growth conditions, the FB Thy-1$^-$c-kit$^+$ population contains approximately one CFC/100 cells plated (Figure 6). This number is comparable with recent data showing that ~3% of Lin$^-$c-kit^{low} cells in bone marrow generate colonies in methyl cellulose assays (Katayama *et al.*, 1993). Although these developmental properties of FB Thy-1$^-$c-kit$^+$ cells suggest a multipotent progenitor cell in fetal blood, it cannot be excluded that the FB Thy-1$^-$c-kit$^+$ population contains separate lineage-committed precursors rather than clonal stem cells. Whether or not the FB Thy-1$^-$c-kit$^+$ population contains precursors of the adult type bone marrow stem cell remains to be determined.

Finally, staining of fetal blood leukocytes for Thy-1 versus c-kit has led to the identification of yet another cell subset (FB Thy-1^{low}c-kit^{high}; Figure 1B and C). The FB Thy-1^{low}c-kit^{high} population expresses strong developmental potential for myeloid lineages because it responds, at frequencies 2- to 3-fold higher than the FB Thy-1$^-$c-kit$^+$ population, to myeloid growth conditions (not shown). Thus, the FB Thy-1^{low}c-kit^{high} population is clearly distinct from the identified pro-thymocytes (FB Thy-1$^+$c-kit^{low}). Future experiments will address further the developmental properties of this fetal blood population.

Progenitor pathways leading to thymopoiesis

Based on the data presented here, fetal blood pro-thymocytes could be placed in the 'hematopoietic tree' 'downstream' from HSC and, importantly, 'upstream' from the earliest thymocytes identified until now (Figure 8). At present, one can only speculate about the origin of fetal blood pro-thymocytes in ontogeny (Figure 8). 'T lineage-promoting microenvironments', other than the thymus, have not been identified in the fetus. Pro-thymocytes could have a direct precursor in fetal liver, gut or omentum. The omental rudiment at day 13 of gestation contains Thy-1$^+$ lymphoid cells (Kubai and Auerbach, 1983) as well as progenitors for Ly1$^+$ B lineage cells (Solvason *et al.*, 1991). Whether pro-thymocyte commitment is induced in these tissues or occurs as a result of a cell autonomous

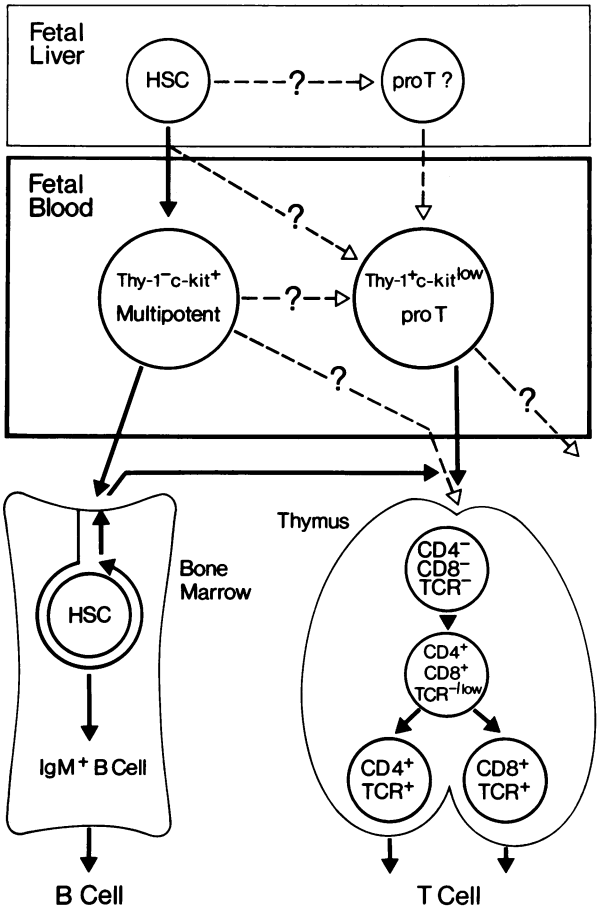

Fig. 8. Model for prethymic T cell development. Fetal blood Thy-1$^+$c-kit^{low} cells fulfil the criteria of T lineage-committed pro-thymocytes. While this population is capable of generating efficiently a single wave of thymopoiesis and subsequently mature T cells, it lacks the potential to generate B lymphocytes and myeloid and erythroid lineages. Fetal blood pro-thymocytes could have a direct precursor in fetal liver or adult bone marrow, or may be generated from multipotent precursors *in situ* in the circulation. In addition to pro-thymocytes, fetal blood contains a multipotent hematopoietic progenitor population (Thy-1$^-$c-kit$^+$) capable of generating T and B cells as well as myeloid and erythroid lineages. Whether or not multipotent FB Thy-1$^-$c-kit$^+$ are capable of direct thymic homing from the circulation remains to be determined. This pluripotent population could be released from yolk sac or fetal liver into the circulation, and may colonize the bone marrow in late gestation.

developmental program is unknown. While fetal blood pro-thymocytes clearly express the potential to generate efficiently thymus-derived αβ T cells, it remains to be determined whether other T cell lineages (extrathymic αβ T cells and intrathymic and extrathymic γδ T cells) and/or natural killer cells can also be pro-thymocyte-derived.

The identification of a multipotent FB Thy-1$^-$c-kit$^+$ cell population in the circulation at mid- to late gestation raises questions about its origin and destination. At day 15.5 in gestation, hematopoiesis is still ongoing in fetal liver (Nicola *et al.*, 1981; McKearn *et al.*, 1985). Thus, it is conceivable that FB Thy-1$^-$c-kit$^+$ cells are fetal liver-derived and may in late gestation colonize, among other hematopoietic tissues, the bone marrow from the circulation (Figure 8). This hypothesis is supported by the fact that the number of c-kit$^+$ cells in peripheral blood in mice declines to undetectable level around birth (unpublished observation).

Multipotent progenitor activity, although at very low levels, has been recovered from the adult (Papiernik *et al.*, 1988; Wu *et al.*, 1991a; Matsuzaki *et al.*, 1993) and early fetal thymus (Péault *et al.*, 1994). However, the fact that HSC activity is difficult to recover from the thymus raises doubts as to the dominance of a direct HSC pathway seeding the thymus. With the identification of pro-thymocytes in fetal blood one can speculate that a major route of thymopoiesis is provided by the pro-thymocyte pathway (Figure 8). Nevertheless, the presence of HSC in the thymus suggests that the extrathymic conversion from HSC to pro-thymocytes may not be essential for HSC seeding of the thymus. For further expansion, receptor gene rearrangement and maturation of pro-thymocytes into T cells, the thymic environment is, however, indispensable (Figure 8). The identification of two cellular compartments in fetal blood which differ by at least one critical parameter, T lineage commitment, could provide a useful basis for the molecular analysis of this earliest stage in T lymphocyte ontogeny.

Materials and methods

Mice

C57Bl/6 (Ly5.1) mice were obtained from Olac, France. Breeding stocks of B6Ly5.2- and RAG-2-deficient mice were kindly provided by Drs Clarence Reeder (NCI, Frederick, MD) and F.Alt (The Children's Hospital, Boston, MA), respectively. Mice were maintained at the animal facility at the Basel Institute under specific pathogen-free conditions. Liver, blood and thymocytes were isolated from fetal mice obtained from timed-pregnant C57Bl/6 or C57Bl/6 (nu/nu) mice. The day of the vaginal plug was counted as day 1 of pregnancy.

Monoclonal antibodies

The following primary antibodies were used in this study: phycoerythrin (PE)-coupled 5a-8 (anti-Thy-1; Caltag, San Francisco, CA), biotinylated ACK-4 (anti-c-kit; Ogawa *et al.*, 1991), FITC-labeled 145-2C11 (anti-CD3; Leo *et al.*, 1987), biotinylated H57-597 (anti-pan TCRαβ; Kubo *et al.*, 1989), FITC-labeled 3A10 (anti-pan TCRγδ; Itohara *et al.*, 1989), PE-coupled GK1.5 (anti-CD4; Dialynas *et al.*, 1983; Becton Dickinson, Mountain View, CA), Red613-coupled 53-6.7 (anti-CD8; Ledbetter and Herzenberg, 1979; Gibco BRL, Gaithersburg, MD), PE-coupled RA3-6B2 (anti-B220; Coffman and Weissman, 1981; Caltag), biotinylated LO-MM (anti-IgM; Caltag), FITC-conjugated 104-2.1 (anti-Ly5.1; Shen, 1981) and FITC-labeled A20-1.7 (anti-Ly5.2; Shen, 1981) (both hybridomas kindly provided by Dr S.Kimura, Sloan-Kettering Cancer Center, New York, NY). Second-step reagents were streptavidin–PE, streptavidin–FITC (both from Southern Biotechnology, Birmingham, AL) and streptavidin–APC (Molecular Probes, Eugene, OR). mAbs were grown as culture supernatants and, depending on the species and isotype, purified on protein A or protein G Sepharose (Pharmacia LKB, Uppsala, Sweden). mAbs were biotinylated using NHS-LC-Biotin (Pierce, Rockford, IL) following the manufacturer's recommendations and FITC-labeled using fluorescein-5-isothiocyanate (FITC 'Isomer I'; Molecular Probes) using standard procedures (Johnson and Holborow, 1986). Following the labeling procedures, the specific reactivity of mAbs was analyzed by FACS and the optimal antibody dilutions were subsequently used.

Isolation of fetal cells

Fetal mice were removed from timed-pregnant mice for the isolation of fetal liver, thymus or blood. To obtain fetal blood, the closed uterus was removed from the mother and washed three times in 10 ml PBS to remove completely maternal blood. The uterus wall was subsequently disrupted under a dissection microscope, the fetus was removed from the uterus without interrupting the umbilical cord, positioned on its back, and jugular veins and cervical arteries were cut with micro-dissection forceps. Approximately 20 μl of fetal blood was collected from each fetus using a heparinized pipette tip and diluted into 10 ml of PBS/5% FCS containing 100 U/ml heparin (Roche, Basel, Switzerland) on ice. Total fetal blood was filtered once through a nylon mesh (Spectrum,

Houston, TX), washed once in PBS/5% FCS and resuspended in 4 ml PBS/5% FCS. To enrich the leukocytes from total fetal blood, density gradient centrifugations were performed. Optimal enrichment of fetal leukocytes, as defined by expression of the common leukocyte antigen CD45, was found at a density of $\rho \geq 1.056$ g/ml. Total blood in 4 ml PBS/5% FCS was layered onto a single-step gradient at a density of 1.056 g/ml corresponding to 60% Percoll (Pharmacia, Uppsala, Sweden) prepared according to the manufacturer's instructions. Gradients were centrifuged for 40 min at 400 *g* at 20°C. Cells were recovered from the interphase of the gradient and washed three times in 12 ml PBS/5% FCS. One fetus at day 15.5 contains ~4500 pro-thymocytes (see text), from which ~10% could be recovered per fetus after the final purification by cell sorting (see below).

Immunofluorescence staining, analysis and cell sorting

For further purification of density gradient-enriched fetal blood subpopulations, cells were purified by FACS. To this end, cells were incubated with 500 μg/ml normal mouse IgG to block non-specific antibody binding and subsequently stained with PE-coupled anti-Thy-1 mAb (1:50 diluted), FITC-labeled anti-CD3 mAb (5 μg/ml) and biotinylated anti-c-kit mAb (5 μg/ml) followed by streptavidin–APC (1:200). All incubations were performed in PBS/5% FCS for 15–30 min on ice. Subsequently, cells were washed in PBS/5% FCS and separated into FB Thy-1$^+$c-kit^{low}CD3$^-$, FB Thy-1$^+$c-kit$^-$CD3$^+$ or FB Thy-1$^-$c-kit$^+$ subpopulations at a flow rate of ~1500 cells/s using a FACSTAR cell sorter (Becton Dickinson). Sorted cell populations were re-analyzed for their purity and were found to be >99% pure. For phenotypic analysis, fresh fetal blood leukocytes, adult thymocytes, lymph node cells and bone marrow cells were obtained from timed fetal mice, or at the indicated times after cell transfer from recipient mice, and stained with mAbs as indicated in the figure legends. $5–10\times10^5$ cells from single cell suspensions were incubated with purified mAbs at 5–10 μg/ml in PBS/5% FCS for 15–30 min on ice and washed once in PBS/5% FCS. Flow cytofluorometric analysis was performed on a FACscan (Becton Dickinson) after gating on viable cells. Fluorescence data are displayed as logarithmic histograms or dot plots using LYSYS software (Becton Dickinson).

Cell transfers in vivo

Intrathymic injections were performed as described (Goldschneider *et al.*, 1986; Rodewald *et al.*, 1992). Briefly, 4–6 week-old mice were irradiated with 600 rad using a ^{60}Co γ-irradiation source (Atomic Energy of Canada Ltd, Ottawa, Canada). Within 3 h after irradiation, mice were anesthetized with Avertin (2.5% solution, 10 μl/g body weight) as described (Hogan *et al.*, 1986). For intrathymic injections, mice were positioned on their backs, a small incision (<5 mm) made in the skin above the sternum without opening the thorax and cells injected into each thymic lobe by positioning the needle for injection from cranio-lateral to caudo-medial and injecting through the thorax wall above the clavicle. Cells ($1–5\times10^4$/thymus) were injected using a 1 ml syringe (Insulin Syringe, Becton-Dickinson) placed into a Tridak Stepper (Tridak, Brookfield, CT) in 20 μl PBS with 5% FCS per lobe. The skin wound was closed using surgical metal clips.

For the analysis of T and B cell development *in vivo*, 6–10 week-old RAG-2$^{-/-}$ recipient mice were irradiated with 400 rad and subsequently injected intravenously into the lateral tail vein with purified fetal blood populations resuspended in 200 μl PBS/5% FCS. Injected cell numbers are indicated in the text.

In vitro hematopoietic differentiation assays

B cell precursor potential *in vitro* was determined as described (Rolink *et al.*, 1993). Briefly, titrated numbers of fetal blood precursors were cultured in SF medium (Rolink *et al.*, 1993) on a feeder layer of PA6 cells (Kodama *et al.*, 1982) at 2×10^4 cells/ml in the presence of 50–200 U recombinant IL-7 for 10 days. Developing pre-B cell colonies were counted through an inverted microscope. Individual representative colonies were harvested and further analyzed phenotypically by flow cytometry.

Myeloid and erythroid precursor potential was analyzed by methyl cellulose assay (Iscove and Schreier, 1979). Titrated numbers of isolated fetal blood precursors were cultured in 35 mm Petri dishes in 1 ml Iscove's-modified Dulbecco's medium (Gibco-BRL) with 10% FCS and cytokines IL-1, IL-3, GM-CSF, stem cell factor (SCF), erythropoietin and transferrin as described (Wiles, 1993). Colonies were counted after 10 days of culture. Individual colonies were harvested, spun onto glass slides and differentially stained (Giemsa–May–Grünwald staining with Diff Quik, Baxter, Switzerland) according to the manufacturer's recommendations.

PCR

Genomic DNA from various tissues was isolated from fetal C57Bl/6 mice. Single-cell suspensions from fetal liver, fetal blood and fetal thymus were washed in cold PBS, resuspended in cold PBS/0.5% NP-40 and incubated on ice for 15 min. Nuclei were spun down, resuspended in 0.5 ml TNE (50 mM Tris, 100 mM NaCl, 1 mM EDTA) with 0.5 mg/ml proteinase K and 1% SDS, and incubated overnight at 37°C. Genomic DNA was extracted once with TE-saturated phenol and once with chloroform and precipitated with 0.3 M NaOAc (pH 5.9) and ethanol. PCR was carried out as described (Anderson *et al.*, 1992) using ~100 ng of DNA template, 360 ng/reaction of primers and 2.5 U of *Taq* polymerase (Perkin Elmer Cetus, Norwald, CT) in 50 μl final volume. The PCR primer recognizes sequences 5′ of the Dβ2.1 [5′-GTAGGCACCTGTGGGGAAGAAACT-3′ corresponding to bp 119–142 in Siu *et al.* (1984)] and 3′ of the Jβ2.7 [5′-TGAGAGCTGTCTCCTACTATCGATT-3′ corresponding to bp 1225–1250 in Chien *et al.* (1984)] elements, respectively. The samples were denatured (94°C, 1 min), annealed (63°C, 2 min) and extended (72°C, 2 min) for 35 cycles. Aliquots from each sample were size fractionated on a 1.2% Tris–Borate–EDTA agarose gel, denatured in 0.25 M HCl and blotted onto a zeta probe membrane (Biorad) in 0.4 M NaOH. Blots were then hybridized with an oligonucleotide probe spanning the Dβ2.1 element (5′-ATTGTGGGGACTGGGGGGGC-3′) using standard methods (Sambrook *et al.*, 1989).

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