Phenotypic and Functional Analysis of T-Cell Precursors in the Human Fetal Liver and Thymus: CD7 Expression in the Early Stages of T- and Myeloid-Cell Development

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It has been proposed that the CD7 molecule is the first antigen expressed on the membrane of cells committed to the T-cell lineage during human fetal T-cell ontogeny. To further identify the pre-T cell subpopulation that migrates to the thymus early in ontogeny, we analyzed the phenotypic and functional characteristics of the fetal liver populations separated on the basis of CD7 expression. Three populations expressing different levels of CD7 were observed: CD7^bright, CD7^dim, and CD7^−. A CD7^bright population depleted of mature T, B, and myeloid cells (lineage negative, lin^−) and mostly composed of CD56^+CD34^− natural killer cells did not mature into T cells in a fetal thymic organ culture (FTOC) assay and was devoid of myeloid progenitors in a clonal colony-forming cell assay. In contrast, the CD7^−/dim CD34^+lin^− populations were capable of differentiating into phenotypically mature T cells after injection into FTOC and contained early myeloid progenitors. Here we phenotypically compared the fetal liver CD7 populations with the most immature fetal thymic subset that differentiated in the FTOC assay, namely the triple negative (TN, CD3^−CD4^−CD8^−) thymocytes. Fetal TN lin^− expressed high levels of CD34 marker and were further subdivided by their expression of CD1 antigen, because CD1^+TN thymocytes express higher levels of CD34 antigen compared with CD1^−TN cells. CD1^+TN thymocytes are characterized by expressing high levels of CD2, CD7, and CD34 markers and null levels of CD6, CD10, and CD28 molecules. We could not find fetal liver pre-T cells with a phenotype equivalent to that of TN thymocytes. Our data show that CD7 does not necessarily identify T-cell precursors during fetal T-cell development and strongly support the hypothesis that the acquisition of early T-cell markers as CD2, CD28, and CD5 molecules on the cell surface of T-cell progenitors takes place intrathymically.

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During human fetal development, thymic T-cell differentiation is initiated at 7 to 9 weeks of gestation, when the first waves of T-cell progenitors start to populate the thymic rudiment.1 The liver is the major hematopoietic organ in the human fetus from the 6th to the 22nd gestational week.2 A number of observations have established that the fetal liver constitutes a source of T-cell progenitors. Transplantation of allogeneic fetal liver cells into severe combined immunodeficiency (SCID) patients has resulted in T-cell reconstitution.3 In addition, fetal-liver-derived precursors develop into T cells after transplantation of fetal liver and thymus into SCID mice.4 In recent years, it has been further shown that CD34^+ fetal liver and fetal bone marrow (BM) cells are able to reconstitute thymic fragments partially depleted of endogenous thymocytes and transplanted into SCID mice.5

The precise characterization of early T-cell progenitors generated in the fetal liver, as well as the assessment of their degree of lineage commitment, have not yet been elucidated. The main limitation in the studies on human T-cell differentiation performed so far has been the lack of in vitro techniques that on the one hand resemble the thymic environment and, on the other, demonstrate the maturation of T-cell precursors in a way that completely excludes the outgrowth of mature contaminating T cells. In the mouse, it has been shown that the injection of BM progenitors into a lethally irradiated host or alternatively the intrathymic injection of hematopoietic progenitors results in long-term thymic reconstitution.7 Through the use of a fetal thymic organ culture (FTOC) system, Jenkinson et al.8 showed differentiation of murine T-cell precursors into mature thymocytes, demonstrating that in vivo conditions are not an essential requirement for T-cell development.

Immunohistologic analyses of the fetal liver and thymus have led to the idea that fetal liver cells expressing CD7 on the cell surface and cytoplasmic (cy) CD3 proteins are prethymic T-cell progenitors.9 This conclusion is based on the assumption that the expression of CD3 proteins is exclusively confined to cells committed to the T-cell lineage and on the observation that the earliest lymphoid cells that populate the thymic rudiment at 7 to 9 weeks of gestation express CD7 on their membranes. However, it has been recently reported that CD34^+ fetal liver natural killer (NK) cells express cytoplasmic CD3 δ and ε proteins.10 These findings indicate that the expression of CD3 proteins is not restricted to T cells and suggest a close relationship between T and NK cells. CD56^cyCD3^+ fetal liver cells express CD7 and it is likely that many of the CD7^cyCD3^+ cells previously identified and assumed to be T-cell progenitors are in fact fetal NK cells. However, it cannot be excluded that CD7^bright cyCD3^+ fetal liver cells have T-cell progenitor potential. The purpose of the present study was to phenotypically and functionally characterize T-cell progenitors in the fetal liver. We examined the expression of CD7 on fetal liver cells of 13 to 22 weeks of gestational age. We report the identification of three subpopulations with different levels of expression of the CD7 marker: CD7^bright, CD7^dim, and CD7^−.
The T-cell and myeloid progenitor potential of these subpopulations depleted of mature T, B, NK, and myeloid cells were evaluated by phenotypic characterization, injection into a novel human FTOC system, and growth in myeloid progenitor assays. The phenotypic and functional characteristics of fetal liver T-cell precursors were compared with those of the earliest fetal thymic progenitors that are CD1 \textsuperscript{+} lin\textsuperscript{-} TN (CD3\textsuperscript{+}CD4\textsuperscript{-}CD8\textsuperscript{-}) thymocytes.

**MATERIALS AND METHODS**

**Monoclonal antibodies (MoAbs).** MoAbs against the following markers were used: CD1 (anti-Leu-6), CD2 (anti-Leu-5b), CD3 (anti-Leu-4), CD4 (anti-Leu-3a), CD5 (anti-Leu-9), and CD7 (CD7-6B7) (Caltag Laboratories, South San Francisco, CA), CD8 (anti-Leu-2a), CD10 (anti-CALLA), CD13 (10M13; Amac, Westbrook, ME), CD14 (anti-Leu-M9), CD16 (anti-Leu-11a), CD19 (anti-Leu-12), CD20 (anti-Leu-16), CD28 (anti-Leu-28), CD33 (My9; Coulter, Hialeah, FL), CD33 (anti-Leu-M9), CD34 (anti-HPCA-2), CD45 (anti-Hle-1), CD56 (L185), and anti-HLA-DR. All these MoAbs were used conjugated to fluorocein isothiocyanate (FITC), phycoerythrin (PE), Tricolor (TC), and immunotinted, as indicated in the legends to the figures. Anti-HLA-A2-FITC (CR1 1-35 I) and anti-HLA-A3-FITC (GAP-A3) were obtained from American Type Culture Collection (ATCC, Rockville, MD). All MoAbs are from Becton Dickinson (San Jose, CA), unless otherwise indicated. CD1-RD (anti-CD1 MoAb PE-conjugated) was obtained from Coulter. Unconjugated MoAbs OKT6 (anti-CD1), RPA-T4 (anti-CD4), and RPA-T8 (anti-CD8) were kindly provided by Dr. G. Aversa (DNAX Research Institute). Anti-ICAM-1-PE MoAb was kindly provided by Dr. G. Aversa (DNAX Research Institute). Anti-HLA-A2-FITC (CR1 1-35 I) and anti-HLA-A3-FITC (GAP-A3) were obtained from American Type Culture Collection (ATCC, Rockville, MD). All MoAbs are from Becton Dickinson (San Jose, CA), unless otherwise indicated. CD1-RD (anti-CD1 MoAb PE-conjugated) was obtained from Coulter. Unconjugated MoAbs OKT6 (anti-CD1), RPA-T4 (anti-CD4), and RPA-T8 (anti-CD8) were kindly provided by Dr. G. Aversa (DNAX Research Institute). Anti-ICAM-1-PE MoAb was kindly provided by Dr. G. Aversa (DNAX Research Institute). Streptavidin (SA) conjugated to allophycocyanin (APC) and SA conjugated to TC were purchased from Becton Dickinson and Caltag Laboratories, respectively.

**Cytokines.** Recombinant human c-kit ligand (KL) and recombinant human interleukin-3 (IL-3) were purchased from R&D Systems (Minneapolis, MN) and were used at 20 ng/mL. Purified recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and purified recombinant human IL-6 were kindly provided by Robert Kastelein (DNAX Research Institute). Anti-ICAM-1-PE MoAb was kindly provided by Dr. L. Lanier (DNAX Research Institute). Streptavidin (SA) conjugated to allophycocyanin (APC) and SA conjugated to TC were purchased from Becton Dickinson and Caltag Laboratories, respectively.

**Isolation of fetal liver subpopulations.** Human fetal tissue was obtained with informed consent from Advanced Bioscience Resources Inc (Alameda, CA), in compliance with regulations issued by the state and by the federal government. Gestational age was determined by crown-rump length and ranged from 13 to 22 weeks. Fetal liver was homogenized through a wire mesh in the presence of RPMI containing 10% fetal calf serum (FCS). The cellular suspension was centrifuged over Histopaque (Sigma, St Louis, MO) to eliminate most of the red blood cells. The remaining erythroid cells were removed by negative selection using magnetic beads. Cells were incubated with saturating amounts of anti-glycophorin MoAb (10F7MN, obtained from ATCC) for 30 minutes at 4°C, then washed twice in phosphate-buffered saline (PBS) containing 5 mg/mL bovine serum albumin (BSA), 0.2 mg/mL NaN\textsubscript{3}. The cells were incubated for 20 minutes in the presence of magnetic beads coated with affinity-purified sheep antimouse IgG (Dynabeads; Dynal, Oslo, Norway), and then the glycophorin positive cells were removed with a magnet (Dynal). The remaining cellular population (10% to 25% from total fetal liver cells) was incubated for 10 minutes at 4°C with 10 μg of mouse IgG1 and IgG2a (Sigma) per million of cells to avoid nonspecific binding of the MoAbs used for the sorting of the populations. The cellular suspension was then incubated for 30 minutes at 4°C with the following FITC-conjugated MoAbs: anti-CD3, anti-CD4, and anti-CD8 (to stain T cells), anti-CD13 (to stain granulocytes), anti-CD14 (to stain monocytes), anti-CD16 and anti-CD56 (to stain NK cells), anti-CD19 and anti-CD20 (to stain B and pre-B cells). After washing, the cells were subjected again to negative selection using magnetic beads. The resulting lineage\textsuperscript{-} population was incubated with the MoAbs indicated in the text for each experiment before cell sorting using a FACStar plus (Becton Dickinson). The purity of the sorted populations was greater than 99% on reanalysis, keeping the same instrument settings that were used during sorting.

**Multicolor analysis of fetal thymocytes.** Fetal thymi were homogenized like the fetal livers (see above). The cells were washed twice in RPMI containing 10% FCS before separating subpopulations. Total thymocytes were incubated 30 minutes at 4°C with saturating amounts of anti-glycophorin MoAb, OKT6 (anti-CD1), RPA-T4 (anti-CD4) and RPA-T8 (anti-CD8), anti-CD19, anti-CD14, and anti-CD56 MoAbs. After extensive washing, the cells were incubated with magnetic beads coated with sheep antimouse MoAb (Dynal) and thymocytes expressing lineage markers; CD1, CD4, and CD8 molecules; were magnetically depleted. To evaluate the possible contamination of cells that were still positive for the indicated markers, an aliquot of remaining cells after the magnetic separation was stained with goat antimouse-FITC and analyzed in a FACScan (Becton Dickinson). Only 3.7% to 5.2% of the cells in different experiments expressed either CD1, CD4, CD8, or lineage markers. The cells were stained with anti-CD2-FITC and biotinylated anti-CD3 (Leu-4), -CD4 (Leu-3a), and -CD8 (Leu-2a) MoAbs, followed by staining with SA-APC. Leu-2a and Leu-3a MoAbs were used for the cell sorting because they recognize different epitopes than RPA-T4 and RPA-T8. The cells were stained with the PE-conjugated MoAbs indicated in the text and multicolor analysis of 2 to 3×10\textsuperscript{4} cells was performed on a FACStar plus. The gates were set to contain 99.5% of CD2\textsuperscript{(FITC\textsuperscript{-})} CD3\textsuperscript{-} 4\textsuperscript{-}8\textsuperscript{-} (APC\textsuperscript{-}) cells.

**Immunofluorescence and flow cytometry.** For cell surface phenotype analysis, 2 to 5×10\textsuperscript{4} cells were incubated with PBS supplemented with 2% FCS and 2% normal mouse serum for 10 minutes on ice, followed by 30 minutes' staining with the indicated MoAbs. For two- and three-color phenotypic analysis, the cells were washed twice in PBS containing 5 mg/mL BSA and 0.2 mg/mL NaN\textsubscript{3} further incubated with SA-TC. After two washings, 10\textsuperscript{5} cells were subjected to cyttofluorometric analysis using a FACScan.

**Isolation of fetal TN thymocytes.** Thymocytes depleted of CD1-, CD4-, CD8-, and glycophorin-positive cells were obtained as described above. The cells were then stained with anti-CD3-PE and anti-CD4, -CD8, -CD56, -CD19, -CD14-FITC-conjugated MoAbs. Cell sorting of PE-FITC (TN thymocytes) in a FACStar plus was performed and reanalysis indicated a purity higher than 99%.

**FTOC.** Human fetal thymic pieces, containing 3 to 10 lobules, were placed on nucleopore filters (0.8 μm; Costar, Cambridge, MA) over gelfoam rafts (Uphol, Kalamazoo, MI) and cultured for the indicated number of days in 6-well plates (Becton Dickinson) at 25°C, in an atmosphere containing 5% CO\textsubscript{2} in the presence of Yssel's medium\textsuperscript{11} supplemented with 1% human serum. To further deplete the endogenous thymocytes, the thymic fragments were irradiated at 1,000 rads before injection. After the sorting of the indicated fetal liver subpopulation from an HLA-mismatched donor, each thymic fragment was microinjected with 3×10\textsuperscript{4} cells (1-mm micropipettes; World Precision Instruments, Sarasota, FL) in 0.5 mL of Yssel's medium using a Nikon microinjector (Nikon, Narishige, Japan). Typically, the number of injected pieces ranged from 20 to 45. The injected pieces were placed back on the gelfoam rafts and cultured in Yssel's medium at 37°C in a humidified 5%
CO₂ atmosphere. At the indicated times, 5 to 10 injected thymic pieces, as well as the noninjected controls, were homogenized by gentle pipetting in PBS containing 5 mg/mL BSA and 0.2 mg/mL Na₃C₅H₅O₆. The resulting cellular suspension was subjected to phenotypic analysis.

**High proliferative-potential colony-forming cell (HPP-CFC) assay.** Sorted fetal liver cells were assayed for myeloid progenitors in double-layered agarose cultures as previously described for the growth of primitive murine hematopoietic progenitors.15 Five hundred or 1,000 sorted fetal liver cells were cultured per culture dish (60 x 15 mm; Becton Dickinson) in the presence of either GM-CSF + IL-3 or GM-CSF + IL-3 + IL-6 + KL. After 2 weeks of growth at 37°C in a humidified 5% CO₂ atmosphere, the clonal cultures were scored for the presence of HPP-CFC and low proliferative-potential colony-forming cells (LPP-CFC). HPP-CFC were defined, as by Bradley and Hodgson,16 as precursors that gave rise to colonies greater than 0.5 mm in diameter. LPP-CFC were scored as colonies smaller than HPP-CFC but greater than 50 cells/colony. 

**Delta (Δ)-assay.** The proliferative capacity of early fetal liver progenitors was measured using the Δ-assay as previously described.14 Briefly, sorted fetal liver progenitor populations were grown in parallel clonal agarose cultures, as described above, and in suspension cultures. Both the clonal and suspension cultures were stimulated by the cytokine combination GM-CSF + IL-3 + IL-6 + KL. Duplicate 1-mL suspension cultures were seeded with 5 x 10³ sorted fetal cells and grown in a humidified atmosphere containing 5% CO₂ in air at 37°C. After 7 days of growth in liquid cultures, the number of suspension culture generated colony-forming cells was determined in secondary clonal cultures. The data are presented as the total number of secondary colony-forming cells (HPP-CFC and LPP-CFC) per 1 x 10⁵ seeded fetal liver cells.

**Statistics.** Significant differences between the number of primary or secondary colony-forming cells measured in different CD7 subpopulations were tested using the two-tailed Student's t-test. The numbers of progenitors observed within separate experiments were considered significantly different when P < 0.05.

**RESULTS**

**Phenotypic and functional characterization of fetal thymic T-cell progenitors.** To address the question of which cells generated in the liver migrate and populate the thymus during fetal life, we first carefully screened the fetal thymus to determine the phenotypic features of the most immature intrathymic pre-T cells subset. To this purpose, we performed three-color immunofluorescence analysis of freshly isolated total thymocytes partly depleted by magnetic beads of CD1⁺, CD4⁺, CD8⁺, and CD5⁺ (CD19, CD56, and CD14) cells. We analyzed the phenotype of the cells expressing CD2 (in FL.1) but negative for CD3, CD4, and CD8 markers (in FL.3) with PE-conjugated MoAbs. Figure 1 shows the representative results of one of four experiments using fetal thymus from a fetus of 19 weeks of gestational age. More than 90% of the fetal CD1⁺lin⁻ TN thymocytes expressed CD45, CD34, CD7, and CD5 surface markers. HLA-DR was found to be positive in about half of the CD1⁺ lin⁻ TN thymocytes. In addition, most of these cells expressed dull levels of CD28. CD33 as well as ICAM-1 were expressed in a very dull fashion in a low proportion of the TN thymocytes. Our results indicated that the phenotypic profile of fetal TN thymocytes essentially did not differ from that already reported in postnatal TN thymocytes.16-17 In some experiments in which the number of acquired cells was higher than 4 x 10⁵ per staining, it was possible to detect a very small fraction of TN thymocytes (1% to 3%) that did not express CD2 on their surface (data not shown). By three-color analysis, we observed that CD1⁺lin⁻ TN thymocytes expressed high levels of CD34 (data not shown) and they may represent the earliest pre-T cell population in the fetal thymus, as has been proposed for the postnatal thymus.18-19

We have recently developed a novel human FTOC that allows the differentiation of postnatal CD34⁺ TN thymocytes containing both CD1⁺ and CD1⁻ cells. To determine the potential to develop into T cells of fetal CD1⁺lin⁻ TN thymocytes, we microinjected this population into FTOC. HLA-A3⁺ thymic pieces (21 weeks of gestational age) were depleted of endogenous thymocytes as described in Materials and Methods. CD1⁺lin⁻ TN thymocytes were sorted from an HLA-A3⁺ fetal thymus of 19 weeks of gestational age. The phenotypic analysis of the freshly isolated CD1⁺lin⁻ TN thymocytes confirmed the previous observations obtained by three-color analysis, because this population expressed CD34 (90.1%), CD7 (94.4%), CD2 (95.7%), CD5 (90.5%), and CD28 (79.8%). Interestingly, most of the CD1⁺lin⁻ TN thymocytes (57.4%) were found to be positive in a dull fashion for CD10 (CALLA). The purity of the sorted population was 99.9% after reanalysis and 3,000 cells were microinjected per thymic fragment. After 19 days in culture, the thymic pieces were homogenized, recovering a threefold increase in cell number relative to the number of injected cells with an 89% viability. This cellular suspension was subjected to immunofluorescence and flow cytometry (Fig 2). The forward versus side scatter plot shows the presence of a population with a lymphoid-like pattern (not observed in the noninjected controls, data not shown). Staining with anti–HLA-A3 specific MoAb indicated that the gated lymphoid population was 90% donor-derived. The progeny of CD1⁺lin⁻ TN thymocytes after FTOC included a large majority of CD4⁺ cells and about half of thymocytes expressed CD3 on the cell surface. The CD4/CD8 plot indicates that most of the cells bore a double-positive phenotype (CD4⁺CD8⁺), most of them expressing the CD1 molecule (data not shown). By three-color analysis, we observed a small percentage of CD3⁺CD4⁺CD8⁻ single positive thymocytes, whereas no CD3⁻CD4⁻CD8⁻ single positive thymocytes were recovered in this particular experiment. The proportion of donor-derived (HLA-A3⁺) cells expressing low to negative levels of CD3 and CD4 markers (8%) was higher than that observed in freshly isolated fetal thymus (0.5% to 2%). These data indicate that fetal CD1⁺lin⁻ TN thymocytes contain T-cell precursors that develop into CD1⁺ double-positive (DP) and single-positive (SP) thymocytes, although the development of thymic T-cell progenitors was not completed at day 19 of FTOC.

**CD7 defines several fetal liver subpopulations.** Once the fetal thymic population containing T-cell progenitor potential had been phenotypically and functionally characterized, we performed a phenotypic analysis to investigate the presence of T-cell-committed progenitors in the fetal liver. Based on the hypothesis that fetal liver prethymic T-cell precursors may express CD7 on the cellular membrane,18 we analyzed the expression of this molecule on fetal liver
cells. The highly erythropoietic potential of the human fetal liver has been reported. Consistent with this finding was our observation that most of the cells recovered after centrifugation over density gradients are committed to the erythroid lineage and express glycophorin, thus making the phenotypic analysis of nonerythroid fetal liver cells difficult without the further depletion of erythroid committed cells. To deplete the erythroid-lineage committed cells, the fetal liver cellular suspension was incubated with antiglycophorin MoAb. Cells expressing glycophorin antigen were then negatively selected with magnetic beads. Glycophorin− fetal liver cells (10% to 25% of total fetal liver cells) from donors between 13 and 22 weeks of gestational age were screened for CD7 expression. The results of a representative experiment are depicted in Fig 3, where glycophorin− fetal liver cells (16 weeks of gestational age) were stained with anti−CD7-PE MoAb. It is important to point out that fetal liver cells bind IgG in a nonspecific manner through high Fc receptor expression (data not shown). CD7bright, CD7dull, and CD7−. As indicated in Fig 3, 62% of the total glycophorin− fetal liver cells expressed CD7 antigen. Among the CD7+ cells, 11% and 52% expressed bright and dull levels of CD7, respectively. The remaining 37% of fetal liver cells are negative for CD7 expression. These three CD7 subpopulations were only observed when CD7 MoAb was conjugated with PE. Due to the higher sensitivity of the PE stainings compared with those performed with FITC-conjugated MoAbs, the phenotypic profile of fetal liver cells stained with FITC-CD7 indicated the presence of positive and negative populations without resolving the positive ones into bright and dull subsets.

The correlation of CD7 intensity of expression and some markers that define distinct hematopoietic lineages is shown in Fig 4. Most of the CD7bright cells coexpressed the NK cell surface marker CD56. Functional NK cells have been reported to be present very early in ontogeny in the fetal liver at a relatively high percentage. CD56 has been recently identified as the neural cell adhesion molecule (NCAM-1) and it is expressed on the membrane of several

**Fig 1.** Three-color analysis of fetal thymocytes. (A) Thymocytes depleted of CD1+, CD4+, CD8+, and lin− cells (CD19+ CD14+ and CD56+) as described in Materials and Methods were stained with anti−CD2-FITC and biotinylated anti−CD3, anti−CD4, and anti−CD8, followed by incubation with SA-APC. The gate was set to contain 99.5% of CD2+(FITC+) CD3−CD4−CD8−(APC−) thymocytes. (B) Cells, 2 to 3 × 10^6, for each individual staining with the indicated PE-conjugated MoAbs, were acquired for multicolor analysis on a FACStar plus. The percentage of positive cells over isotype-matched control MoAbs is represented.
cellular types in hematopoietic, muscle, and neural tissues. Expression of CD56 in the CD7- compartment is likely caused by the presence of nonhematopoietic cells in the cellular suspension of unseparated fetal liver. CD3+ T cells were observed in the total fetal liver, most of them expressing bright to moderate levels of CD7. CD33+ myeloid progenitors were dull to negative for CD7. Most of the B cells (CD19+ and CD20+) were found in the CD7- compartment, but some coexpressed dull levels of CD7. The majority of pre-B cells (CD10+) were CD7-. Few monocytes (<5% of CD14+ cells) and granulocytes (<3% of CD13+ cells, data not shown) were detected in the human fetal liver.

To further phenotypically characterize the three CD7 fetal liver subsets, we isolated highly purified populations of CD7bright, CD7dull, and CD7- cells by cell sorting for subsequent surface-marker analysis. For the isolation of the CD7bright population, glycophorin-depleted fetal liver cells were stained with anti-CD7-PE MoAb and FITC-conjugated anti-CD3, anti-CD13, anti-CD14, anti-CD19, and anti-CD20. For the sorting of the CD7dull and CD7- subsets, the same protocol was followed, but FITC-conjugated anti-CD56 MoAb was added to stain mature NK cells. After cell sorting, the three subpopulations were subjected to fluorescence-activated cell sorter (FACS) analysis (Table 1). The cellular recovery after sorting of the three CD7 populations were (n = 5): 3.5% ± 0.5% for CD7dull, 0.8% ± 0.2% for CD7-, and 0.5% ± 0.2% for CD7bright. These percentages are referred to the number of glycophorin+ fetal liver cells before sorting. The CD7bright fetal liver cells exhibited low to undetectable levels of CD33, CD34, CD4, and CD5 markers. Both CD2 and CD8 molecules defined positive and negative subpopulations within this subset. Moreover, most of these cells (96% to 98% in several experiments) expressed CD56 on the cell membrane and displayed NK activity.

In contrast to CD7bright fetal liver cells, both CD7dull and CD7- populations expressed high levels of HLA-DR and of the stem cell marker CD34 (Table 1). Furthermore, CD7- cells were enriched in CD34+ bright cells relative to CD7dull cells (data not shown). The expression of other markers present on lineage-committed immature cells was similar in CD7- and CD7dull populations. The expression of the myeloid progenitor marker CD33 in most of the CD7dull and CD7- cells suggests the commitment of these populations to the myeloid lineage. Interestingly, few CD7dull and CD7- cells expressed the early lymphoid marker CD10. Other pre-T cell markers present on fetal TN thymocytes were either negative (like CD5) or almost undetectable (like CD2 and CD28). Although for the T-cell lineage depletion only anti-CD3 MoAb was included, the CD7dull and CD7- populations did not express other T-cell markers such as CD4 and CD8.

**Differentiation potential of CD7 fetal liver subpopulations.** Our data indicate that fetal liver cells, depleted of lineage markers, and expressing low and negative levels of CD7, showed an indistinguishable phenotypic profile that contrasted with that of CD7bright cells. This observation prompted us to investigate the T-cell progenitor potential of CD7bright and the whole population comprised of CD7+ and CD7dull cells (CD7dull). It has been recently shown that CD34+ fetal liver and BMcells contain T-cell precursors (and our own observations). Because CD7bright cells did not express the CD34 marker, it was not...
were not clonogenic, thus indicating the absence of contamination. CD7\(^{-}\) or CD7\(^{+}\) cells did not express the CD1 molecule, which was negative in the fetal liver starting population (A.B., H.S., manuscript in preparation). Interestingly, a high number of cells expressing low levels of CD34 (42.3%) were observed in the donor FTOC-derived cells compared with normal fetal thymocytes (1.5% to 2.5%) (Fig 5A). We have recently shown that CD34, a marker present in 2% of total thymocytes, is expressed at high levels on CD3\(^{-}\)CD4\(^{-}\)CD8\(^{-}\) and on CD3\(^{+}\)CD4\(^{+}\)CD8\(^{-}\) thymocytes. Therefore, our data indicate a higher proportion of an immature CD34\(^{+}\) population at day 25 in FTOC as compared with normal fetal thymus. The suggestion of an immature population in FTOC was confirmed by three-color analysis (Fig 5B), where a fivefold increase of CD3\(^{+}\)CD4\(^{+}\) immature thymocytes was observed compared with fresh normal fetal thymocytes. In addition, fewer CD3\(^{+}\) cells (57.7%) were present in FTOC relative to normal thymocytes (86%).

To better characterize the emergent population of thymocytes derived from fetal liver precursors in FTOC, three-color analyses were performed (Fig 6). The percentage of DP CD4\(^{+}\)CD8\(^{+}\) thymocytes was reduced compared with a normal fetal thymus (47.2% and 66%, respectively), but CD1 and CD3 expression on DP thymocytes was quite comparable with freshly isolated fetal thymus. The percentage of SP CD4\(^{+}\) and CD8\(^{+}\) thymocytes varied in different experiments, resembling the distribution found in the normal thymus, where the SP CD4\(^{+}\) population is always larger than SP CD8\(^{+}\). Most SP CD4\(^{+}\) thymocytes generated in FTOC express the CD1 marker (Fig 6), and 21.8% coexpress CD3 on cell surface. A striking difference relative to the fresh fetal thymus was a 9- to 10-fold increase in the proportion of double-negative (DN) CD4\(^{-}\)CD8\(^{-}\) thymocytes (16%), thus again confirming the immature stage of thymocyte development in FTOC. As observed in the fresh thymus, CD1 was found to define positive and negative subpopulations in the FTOC-derived DN compartment, which is mostly comprised of CD3\(^{+}\) cells (Fig 6).

**T-cell differentiation potential of CD7\(^{-}\) and CD7\(^{+}\) fetal liver cells.** The requirement for the expression of the CD7 molecule on fetal liver progenitors was examined using FTOC (Fig 7). CD34\(^{+}\)CD7\(^{-}\)lin\(^{-}\) and CD34\(^{+}\)CD7\(^{+}\)lin\(^{-}\) fetal liver cells were sorted from a 19-week-old HLA-A3\(^{+}\) specimen. The purity of the sorted populations was 99.5% and 99.8%, respectively. HLA-A3\(^{-}\) fetal thymic fragments (19 weeks) were depleted of endogenous thymocytes and injected with 5 \(\times\) \(10^5\) sorted cells per thymic piece. Thymic pieces were homogenized after 7 and 18 days in culture, and with CD7\(^{-}\) CD34\(^{+}\) lin\(^{-}\) cells are depicted in Fig 5. After 25 days in FTOC, these thymic pieces contained a fourfold to fivefold increase in the number of cells relative to the number of injected cells, and most of the thymocytes (>98%) expressed the donor HLA-A2\(^{+}\) phenotype with an 82% viability. Recipient-derived cells (HLA-A2\(^{-}\)) bearing a CD3\(^{+}\)CD4\(^{-}\)CD8\(^{-}\) phenotype were observed in the cultures at early time points (7% to 15% after 15 days in FTOC) but were not detectable at day 25 (data not shown). Two-color analysis of the thymocytes derived from FTOC showed that, as in the normal fetal thymus, a large majority of the cells expressed the CD1 molecule, which was negative in the fetal liver starting population (A.B., H.S., manuscript in preparation). Interestingly, a high number of cells expressing low levels of CD34 (42.3%) were observed in the donor FTOC-derived cells compared with normal fetal thymocytes (1.5% to 2.5%) (Fig 5A). We have recently shown that CD34, a marker present in 2% of total thymocytes, is expressed at high levels on CD3\(^{-}\)CD4\(^{-}\)CD8\(^{-}\) and on CD3\(^{+}\)CD4\(^{+}\)CD8\(^{-}\) thymocytes. Therefore, our data indicate a higher proportion of an immature CD34\(^{+}\) population at day 25 in FTOC as compared with normal fetal thymus. The suggestion of an immature population in FTOC was confirmed by three-color analysis (Fig 5B), where a fivefold increase of CD3\(^{+}\)CD4\(^{+}\) immature thymocytes was observed compared with fresh normal fetal thymocytes. In addition, fewer CD3\(^{+}\) cells (57.7%) were present in FTOC relative to normal thymocytes (86%).

To better characterize the emergent population of thymocytes derived from fetal liver precursors in FTOC, three-color analyses were performed (Fig 6). The percentage of DP CD4\(^{+}\)CD8\(^{+}\) thymocytes was reduced compared with a normal fetal thymus (47.2% and 66%, respectively), but CD1 and CD3 expression on DP thymocytes was quite comparable with freshly isolated fetal thymus. The percentage of SP CD4\(^{+}\) and CD8\(^{+}\) thymocytes varied in different experiments, resembling the distribution found in the normal thymus, where the SP CD4\(^{+}\) population is always larger than SP CD8\(^{+}\). Most SP CD4\(^{+}\) thymocytes generated in FTOC express the CD1 marker (Fig 6), and 21.8% coexpress CD3 on cell surface. A striking difference relative to the fresh fetal thymus was a 9- to 10-fold increase in the proportion of double-negative (DN) CD4\(^{-}\)CD8\(^{-}\) thymocytes (16%), thus again confirming the immature stage of thymocyte development in FTOC. As observed in the fresh thymus, CD1 was found to define positive and negative subpopulations in the FTOC-derived DN compartment, which is mostly comprised of CD3\(^{+}\) cells (Fig 6).

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the resulting cellular suspension was subjected to FACS analysis. Both CD7<sup>dim</sup> and CD7<sup>-</sup> fetal liver cells were able to reconstitute thymic fragments in three independent experiments, and the acquisition of CD3, CD4, and CD8 T-cell markers in the donor FTOC-derived progeny was observed (data not shown). The percentage of DP thymocytes present in FTOC at day 18 (25% from CD7<sup>+</sup> and 22% from CD7<sup>dim</sup> fetal liver cells) was equivalent to that observed when the combined population (CD7<sup>-dim</sup>) was injected (12% to 47%). The cellular viability at day 18 of the FTOC-derived cells was comparable among recipients injected with CD7<sup>-</sup>, CD7<sup>dim</sup>, and CD7<sup>-dim</sup> cells (range 82% to 89%). However, a difference was noted in the total number of cells recovered from FTOC 18 days after injection with CD7<sup>-</sup> or CD7<sup>dim</sup> cells. Recipients injected with CD7<sup>-</sup> cells had an eightfold increase in cellularity versus a fourfold increase in cellularity in recipients of CD7<sup>dim</sup> cells. Interestingly, the progeny in FTOC of CD7<sup>-</sup> fetal liver cells acquired CD7 expression at early time points in culture. Thirty-nine percent of donor-derived (HLA-A3<sup>+</sup>) were positive for CD7 at day 7 in FTOC.

### Table 1. Phenotypic Analysis of Sorted CD7 Subpopulations in the Human Fetal Liver

<table>
<thead>
<tr>
<th>Population&lt;sup&gt;*&lt;/sup&gt;</th>
<th>CD45</th>
<th>CD34</th>
<th>HLA-DR</th>
<th>CD33</th>
<th>CD56</th>
<th>CD2</th>
<th>CD4</th>
<th>CD5</th>
<th>CD8</th>
<th>CD10</th>
<th>CD28</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD7&lt;sup&gt;dim&lt;/sup&gt; lin&lt;sup&gt;-&lt;/sup&gt;</td>
<td>98 ± 1.8</td>
<td>&lt;1</td>
<td>45 ± 9</td>
<td>&lt;1</td>
<td>95 ± 3</td>
<td>36 ± 8</td>
<td>2.5 ± 1.5</td>
<td>1.5 ± 0.5</td>
<td>60 ± 4</td>
<td>ND</td>
<td>59 ± 4</td>
</tr>
<tr>
<td>CD7&lt;sup&gt;-dim&lt;/sup&gt; lin&lt;sup&gt;-&lt;/sup&gt;</td>
<td>99 ± 0.6</td>
<td>93 ± 3</td>
<td>88 ± 5</td>
<td>82 ± 12</td>
<td>&lt;1</td>
<td>7 ± 2.5</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>2 ± 0.5</td>
<td>3.5 ± 1.8</td>
</tr>
<tr>
<td>CD7&lt;sup&gt;-&lt;/sup&gt; lin&lt;sup&gt;-&lt;/sup&gt;</td>
<td>99 ± 0.7</td>
<td>94 ± 3</td>
<td>87 ± 4</td>
<td>69 ± 6.5</td>
<td>&lt;1</td>
<td>3.5 ± 1.2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1.5 ± 0.7</td>
<td>11 ± 5</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

<sup>*</sup> CD7 populations from fetal livers of 17 to 21 weeks of gestation were >99.5% pure after reanalysis. The cells were stained with FITC-conjugated MoAbs against the indicated surface markers and subjected to cytofluorometric analysis with a FACScan. The results are expressed as mean ± SD of the percentage of positive cells over isotype-matched controls (n = 3).
HLA-A2-FITC

CD3+ THYMOCYTES

CD3- THYMOCYTES

Fig 5. Differentiation of CD34+CD7-/-lin- fetal liver cells in FTOC. CD34+CD7-/-lin- sorted cells, 1 x 10^6, from HLA-A2+ fetal liver were microinjected into individual HLA-A2- thymic fragments and cultured in Yssel's medium. After 25 days, the fragments were homogenized and subjected to two-color (A) and three-color (B) analysis. In (B), the electronic gates were set to contain 97.3% (upper panel right) and 98.8% (lower panel right) of CD3+ and CD3- donor-derived cells, respectively.

(Fig 7), whereas virtually all of the CD7null HLA-A3+ cells expressed CD7 (data not shown). Notably, the acquisition of CD7 expression correlated with the appearance of the CD2 molecule at day 7 and 18 in FTOC (Fig 7).

Myelopoietic potential of fetal liver CD7 subpopulations. To further define the multilineage proliferative capacity of fetal liver CD7 subpopulations, their myeloid growth potential was assessed in both clonal and suspension culture assays (Table 2). CD34+CD7null lin- fetal liver cells did not contain any colony-forming progenitors. In contrast, CD34+lin- fetal liver cells sorted on the basis of moderate or negative levels of CD7 expression contained colony-forming cells with both high and low proliferative potential. The median number of LPP-CFC from four experiments was twofold higher in the CD7null population than in the CD7+ population (P = 0.06 for experiments B through E). Because the lower proliferative capacity of LPP-CFC relative to HPP-CFC is believed to reflect a more mature stage of differentiation, these data suggest that CD7 expression increases with the maturation of myeloid progenitors. Consistent with this hypothesis was the observation that the median number of HPP-CFC was 1.8-fold higher in the CD7+ population relative to the CD7null population. However, the higher number of HPP-CFC found consistently in the CD7+ population relative to the CD7null population was significant in only one experiment. Therefore, we attempted to further resolve differences in the proliferative capacity of the CD7+ and CD7null population using the Δ-assay. The total number of secondary colony-forming cells generated in suspension cultures of CD7+ cells was 1.8- to 3.1-fold greater than the number of secondary progenitors obtained from cultures of CD7null cells. Furthermore, phenotypic analysis of CD34+CD7null cells after 3 days of growth in GM-CSF + IL-3 + IL-6 + KL stimulated Δ-culture showed that 70% of the recovered CD3+ cells expressed dull levels of CD7 (data not shown). Thus, these data suggest that the CD7null fetal liver progenitors appear to have a greater myeloid proliferative capacity than the CD7null population and may represent precursors of CD7null cells.

To further investigate phenotypic differences between CD7null and CD7null populations that might reflect a different stage of development, two- and three-color FACS analyses were performed on freshly isolated glycoporphin-depleted fetal liver cells. It has been shown that CD34+CD38BM cells have a greater proliferative capacity than CD34+CD38null cells when grown on BM stromal layers. Moreover, we have observed that HPP-CFC are contained within the CD34+CD33+CD38 null fraction of fetal liver cells. Figure 8A shows one of three representative experiments of two-color staining of glycoporphin fetal liver cells from a 19-week-old specimen. The results in Fig 8A indicated that
CD38− cells expressed high levels of CD34. This population (CD34bright CD38−) represents the 2.3% of the total glycoprophorin− fetal liver cells. Sorted CD34bright CD38− fetal liver cells were found to contain similar numbers of CD7− and CD7null cells, and similar numbers of HPP-CFC were observed in both CD7 populations consistent with the results presented in Table 2 (data not shown). To study the CD38 expression pattern on CD7 subpopulations, three-color FACS analyses on fetal liver cells were performed. Figure 8B shows that a decrease in CD7 expression among CD34bright cells correlated with a decrease in CD38 expression. The mean intensities of fluorescence for CD38-PE expression among the three gated populations (CD34−CD7bright, CD34+C7null, and CD34−CD7−) were 1,165, 107, and 30, respectively. These results indicate a strong correlation between these two markers in CD34bright fetal liver cells. In conclusion, these phenotypic and functional data show that CD7 expression increases with the differentiation of progenitors along the T- and myeloid-cell differentiation pathways.

DISCUSSION

The nature of the prethymic population present in the human fetal liver has remained unknown in that all the available data have been mostly limited to immunohistologic studies of human fetuses. It has been shown that human CD34+ fetal liver cells contain precursors able to develop into T cells.6,28 In addition, two recent reports provided evidence that fetal liver progenitors can develop into T cells in vitro in the presence of thymic stroma and IL-2,21 or phytohemagglutinin (PHA) and IL-2.32 However, these studies have not addressed the question of whether or not the fetal liver contains cells that are already committed to the T-cell lineage. Previous reports suggested the relevance of membrane CD7 and cyCD3 expression in the definition of human fetal liver progenitor cells committed to the
T-cell lineage. These conclusions were based on the observations that the perithymic mesenchima is stained with anti-CD7 and anti-CD45 MoAbs at 7.5 weeks of gestation, which correspond to the time frame for thymic colonization. These cells were thought to be derived from a CD7+cyCD3+ population present in the human fetal liver. However, direct proof that those cells develop into mature T cells has been lacking. Moreover, recent findings showing that fetal liver NK cells express cyCD3 proteins have opened the possibility that the CD7+cyCD3+ fetal liver cells are not T-cell precursors. This new information prompted us to re-examine the phenotypic and, more importantly, the functional features of CD7+ and CD7- fetal liver cells. In preliminary studies using FITC-labeled anti-CD7 MoAbs, two populations, CD7+ and CD7-, were detected in the fetal liver. In contrast, by using the more sensi-

Table 2. Frequency of HPP-CFC and LPP-CFC Among Different CD7 Populations in the Fetal Liver

<table>
<thead>
<tr>
<th>Experiment</th>
<th>CD7- Primary Colonies</th>
<th>CD7- Secondary Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD7-(cells)</td>
<td>CD7-(cells)</td>
</tr>
<tr>
<td>A</td>
<td>0 ± 0.1</td>
<td>52.0 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>(0 ± 0.1)</td>
<td>(89.3 ± 5.8)</td>
</tr>
<tr>
<td>B</td>
<td>44.0 ± 3.1</td>
<td>55.3 ± 9.3</td>
</tr>
<tr>
<td></td>
<td>(114 ± 1.2)</td>
<td>(64.7 ± 13.0)</td>
</tr>
<tr>
<td>C</td>
<td>96.7 ± 3.7</td>
<td>113 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>(196 ± 5.3)</td>
<td>(107 ± 4.4)</td>
</tr>
<tr>
<td>D</td>
<td>36.3 ± 7.1</td>
<td>44.0 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>(128 ± 6.1)</td>
<td>(82.7 ± 13.7)</td>
</tr>
<tr>
<td>E</td>
<td>44.7 ± 6.4†</td>
<td>103 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>(183 ± 12.7)†</td>
<td>(74.7 ± 5.5)</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.
* Results are presented as the mean ± SE of three cultures.
† Clonal cultures in Experiment A were stimulated by the cytokine combination GM-CSF + IL-3. In all other experiments the growth of fetal liver cells was stimulated by GM-CSF + IL-3 + IL-6 + KL.
‡ Results differ significantly ($P < .05$) relative to the same colony type observed in the CD7- population within the same experimental group.
Fig 8. Multicolor phenotypic analysis of glycophorin- fetal liver cells. Freshly isolated mononuclear glycophorin- fetal liver cells (19 weeks old) were incubated with the indicated MoAbs and subjected to two-color (A) and three-color (B) phenotypic analysis on a FACScan. 6 X 10^6 events of alive gated cells were acquired. In (A), the electronic gate was set to contain 95% of CD34^{bright}CD7^{low} cells. In (B), the electronic gates were set to contain greater than 95% of the CD34^{bright}CD7^{dull}, CD34^{bright}CD7^{dull}, and CD34^{-}CD7^{lo}. The numbers indicate the percentage of positive cells over isotype-matched PE controls.

It has been reported that stem cells isolated from fetal BM are contained in a population characterized by CD34^{high}CD38^{-}. Three-color analysis of total fetal liver cells depleted of glycophorin- cells showed that CD34^{bright}CD38^{-} fetal liver population contained CD7^{dull} and CD7^{dull} in equivalent numbers. In addition, primary myeloid clonal cultures of both CD7^{-} and CD7^{dull} subpopulations gave rise to HPP-CFC. However, the CD7^{dull} subpopulation did contain more cells of the committed progenitor compartment, the LPP-CFC compartment, than did the CD7^{-} subpopulation. In the murine system, HPP-CFC have been well characterized and are considered to be the most primitive compartment of myeloid progenitors detectable in vitro. Likewise, we have also shown that human fetal liver HPP-CFC represent a compartment of primitive progenitors apparently committed to the myeloid lineage because these progenitors are found primarily within the CD34^{CD33^{low}CD38^{-}} fraction of fetal liver cells (Muench MO, Cupp J, Polakoff J, Roncarolo MG, manuscript submitted). The cell recoveries from FTOCs did suggest a higher proliferative potential in the CD7^{-} subpopulation compared with the CD7^{dull} subpopulation. Similarly, the
CD7Negative thymic progenitors had a myeloid proliferative capacity that was greater than that of CD7null cells, as suggested by a greater number of secondary HPP-CFC and LPP-CFC in suspension cultures of CD7Negative cells. Furthermore, we determined that sorted CD34PositiveCD7Negative cells upregulated CD7 expression after 3 days of in vitro growth. In total, these data strongly suggest that with commitment along the T-cell and myeloid differentiation pathways CD7 expression is increased. Therefore, hematopietic stem cells may be CD7Positive and begin to express dull levels of CD7 at an early stage of commitment. It is also possible that the low expression of the CD7 marker does not by itself define a distinct cellular population, but its expression could be, for example, related to the “activation” or cell cycle state of progenitors. Supporting this hypothesis are the reported data of CD7 expression on neoplastic cells that have been classified as undifferentiated leukemias as well as “stem cell leukemias.” In addition, the presence of CD7Negative myeloid precursors in the postnatal thymus and in the fetal liver has been reported. From these data we conclude that the presence of the CD7 molecule on early fetal precursors does not ensure commitment to the lymphoid or myeloid cell lineages. This idea would be compatible with the findings that committed CD3Positive myeloid progenitors (and our observations), as well as some B-cell precursors CD19Negative (and our observations) contain CD7Negative and CD7null cells.

The CD7Bright linNegative population consisted mainly of CD56Positive cells and had no detectable expression of CD34 marker. In contrast to the CD7NegativeCD7null subsets, the CD7Bright linNegative population had no T-cell progenitor activity in FTOC or myeloid progenitor activity in a clonal colony-forming cell assay. To exclude the possibility that the failure of CD7Bright linNegative cells to differentiate into T cells was because only very early progenitors could mature in this system, we examined the phenotype and T-cell precursor activity of highly purified fetal thymic T-cell progenitors (CD1Negative TN thymocytes). The phenotypic profile of fetal CD1Negative TN thymocytes was characterized by high levels of expression of CD2, CD7, and CD34 markers. CD5, CD10, and CD28 molecules were also expressed in CD1Negative TN thymocytes in a dull fashion. These data indicate a high degree of T-cell lineage commitment in this population. Furthermore, fetal CD1Negative TN thymocytes did not differ from their postnatal counterpart population in their expression of CD antigens, suggesting that the thymic T-cell developmental pathway is not different throughout distinct ontogenetic stages in humans. This idea is supported by the fact that fetal thymic fragments can drive the maturation of both fetal (shown here) and postnatal thymic T-cell progenitors.

Despite the similarity of CD7Bright linNegative fetal liver cells and fetal thymic T-cell precursors in the expression of CD2, CD7, CD28, cyCD3, and the lack of expression of CD1 (data not shown), there were a number of fundamental differences between these two populations. Most importantly, fetal thymic T-cell progenitors developed into CD3PositiveDP and into CD3nullSP thymocytes and CD7Bright linNegative fetal liver cells did not. This functional difference is compatible with the fact that in contrast to CD7Bright linNegative fetal liver cells, more than 90% of thymic T-cell progenitors expressed CD34. Recently it has been shown that CD34Positive cells from the fetal liver and BM can reconstitute the fetal human thymus in the SCID-hu system, showing that CD34 is present on T-cell precursors. Combined with the finding that the majority of CD7Bright linNegative are CD34Positive, CD5Positive, and display cytotoxic activity, our results suggest that the reported CD7Positive cyCD3Positive fetal liver cells are comprised mostly of functional fetal NK cells, although the presence of T-cell precursors cannot be completely ruled out.

The distribution of populations of T-cell progeny derived from fetal liver precursors in FTOC follows the one observed in the normal fetal thymus, because most of the cells displayed a CD4PositiveCD8Negative phenotype and CD1 expression increases during the in vitro differentiation. However, there was a fivefold increase in the percentage of CD3NegativeCD4PositiveCD8Negative immature thymocytes and a 9- to 10-fold increase in the proportion of CD4PositiveCD8Negative in FTOC relative to fresh fetal thymocytes. These differences can be a reflection of an incomplete development caused by the immature stage of the injected population. On the other hand, although less prominent, an increase of the same populations was detected when intrathymic T-cell committed progenitors were injected. Therefore, we conclude that the accumulation of thymocytes in the early stages can be a consequence of technical limitations of FTOC, which only allows the growth of thymocytes for 3 to 4 weeks. Another interesting observation was the consistent low percentage of CD3PositiveCD4PositiveCD8Negative cells that were obtained in FTOC, regardless of the starting population. It may reflect a deficient positive selection of CD8Negative mature cells, which does not occur on CD4Positive cells. However, despite this possibility, the observation that recipient-derived cells are mainly CD3PositiveCD4Positive and very few or no CD8Negative mature cells are detected (data not shown), suggests the absence of some signal involved in the maintenance of this particular population.

Analysis of several pre-T-cell markers (present in fetal TN thymocytes) on the linNegative subpopulations showed that there was no “TN-like” population in the fetal liver between 14 and 22 weeks of gestational age. The expression of CD5 and CD28 markers was undetectable in lineage depleted cells. However, CD10 (lymphoid progenitor marker) was present in a small percentage of the CD7Negative population. CD2 was also observed in a dull fashion and in a low proportion of CD7Negative fetal liver cells. These results suggest that the complete commitment to the T-cell lineage takes place intrathymically, once either stem cells or lymphoid committed progenitors migrate to the thymus. These data contrast with the reported existence of a committed T-cell progenitor in the fetal and adult BM. The fetal BM T-cell precursors were reported to be a very minor population, characterized by multicolor flow cytometry, composed of CD34Positive cells with a similar phenotype as TN thymocytes (CD34PositiveCD7NegativeCD2PositiveCD5NegativeLECAM-1Positive). However, a direct proof for the existence of this population, which requires its isolation and subsequent functional analysis, is still lacking. Adult BM CD2PositiveCD7Negative T-cell precursors were not observed in a study by Terstappen et al. However, Tjonnfjord et al described two populations in the adult BM (CD34PositiveCD7Negative and CD34PositiveCD2Positive) capable of dif-
ferentiating into CD3^+CD4^- and CD3^+CD8^+ T cells in the presence of thymic stroma cells. However, this group did not report the development of an intermediate cellular population expressing the CD1 antigen. Thus, whether or not the CD34^+CD7^+ population described by Tjennfjord et al is similar to the CD34^+CD7^null fetal liver population described in the present report is unclear.

Finally, we have shown that human FTOC can be a powerful in vitro system for investigating the early events in T-cell development as well as in searching for prethymic T-cell progenitors. It has been recently reported that human fetal CD4^-CD8^- thymocytes develop into CD4^+CD8^- and mature thymocytes after coculture with mouse thymic lobes. Herein we showed that fetal CD1^+TN thymocytes, as well as fetal liver T-cell progenitors, are capable to differentiate into T cells in a human FTOC system. The advantages of the present study compared with the one mentioned above are several. First, we sought to work with purified sorted populations highly enriched in immature thymocytes that do not give rise to mature cells in regular culture conditions that favor T-cell growth. In this way, we avoided the possibility of contaminating populations and the presence of a mature CD4^-CD8^- thymic population, which may have an as-yet-undetermined influence on T-cell development. Second, the chimeric mouse-human FTOC requires the presence of a poorly characterized source of human thymic stroma cells, normally present in the human-human system. Third, we show here that the human FTOC system is capable of supporting the differentiation of prethymic (fetal liver-derived) T-cell precursors, not reported so far in the chimeric mouse-human FTOC system.

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Phenotypic and functional analysis of T-cell precursors in the human fetal liver and thymus: CD7 expression in the early stages of T- and myeloid-cell development

A Barcena, MO Muench, AH Galy, J Cupp, MG Roncarolo, JH Phillips and H Spits