CD34+CD38dim Cells in the Human Thymus Can Differentiate Into T, Natural Killer, and Dendritic Cells But Are Distinct From Pluripotent Stem Cells

By Pieter Res, Eva Martinez-Cáceres, Ana Cristina Jaleco, Frank Staal, Eric Noteboorn, Kees Weijer, and Hergen Spits

Recently we reported that the human thymus contains a minute population of CD34+CD38dim cells that do not express the T-cell lineage markers CD2 and CD5. The phenotype of this population resembled that of CD34+CD38dim cells present in fetal liver, umbilical cord blood, and bone marrow known to be highly enriched for pluripotent hematopoietic stem cells. In this report we tested the hypothesis that the CD34+CD38dim thymocytes constitute the most primitive hematopoietic cells in the thymus using a combination of phenotypic and functional analyses. It was found that in contrast to CD34+CD38dim cells from fetal liver and bone marrow, CD34+CD38dim cells from the thymus express high levels of CD45RA and are negative for Thy-1. These data indicate that the CD34+CD38dim thymocytes are distinct from pluripotent stem cells. CD34+CD38dim thymocytes differentiate into T cells when cocultured with mouse fetal thymic organs. In addition, individual cells in this population can differentiate either to natural killer cells in the presence of stem cell factor (SCF), interleukin-7 (IL-7), and IL-2 or to dendritic cells in the presence of SCF, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor α (TNFα), indicating that CD34+CD38dim thymocytes contain multi-potential hematopoietic progenitors. To establish which CD34+ fetal liver subpopulation contains the cells that migrate to the thymus, we investigated the T-cell-developing potential of CD34+CD38dim and CD34+CD38+ fetal liver cells and found that the capacity of CD34+ fetal liver cells to differentiate into T cells is restricted to those cells that are CD38dim. Collectively, these findings indicate that cells from the CD34+CD38dim fetal liver cell population migrate to the thymus before upregulating CD38 and committing to the T-cell lineage.

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In human fetal development, T-cell progenitors start to migrate to the thymic rudiment at 8 weeks of gestation.1 The progenitors that migrate to the thymus are initially derived from pluripotent stem cells that reside in the fetal liver.2 Beginning at week 16 of gestational age, stem cells from the liver colonize the bone marrow and by week 22 all thymic progenitors are believed to be derived from the bone marrow. The question whether human thymic immigrants are identical to pluripotent stem cells or whether they are differentiated into more mature cells at the time they enter the thymus has not yet been resolved. Specifically, it is uncertain whether thymic immigrants contain progenitor cells that are committed to develop into T cells but not into other cell lineages. Studies to this question in the mouse have yielded conflicting results. Some of these studies support the notion that the cell that colonizes the thymus is a multipotent progenitor cell.3,4 One recent study, however, provided evidence that commitment to the T-cell lineage (ie, rearrangement at the TCR β locus) occurs before entry into the thymus.5

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MATERIALS AND METHODS

Cell separations. Postnatal normal thymus tissues were obtained from patients of 2 months to 5 years of age who underwent median sternotomy and corrective cardiovascular surgery. Fetal thymic tissues were obtained from elective therapeutic abortions. Gestational age was determined by crown-rump length and ranged from 16 to 18 weeks. The use of this tissue was approved by the medical ethical committee of the Netherlands Cancer Institute and was contingent upon informed consent. To get a single cell suspension, fresh thymus fragments were finely minced and pressed through a stainless steel mesh. Large aggregates were removed, and the cells were washed

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before use. To prepare CD34+ subpopulations, total thymocytes were first incubated with saturating concentrations of anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), generously provided by Dr G. Aversa (DNAX Research Institute, Palo Alto, CA), anti-CD69 (Leu-23, a gift of Dr J.H. Phillips, DNAX), and anti-CD27 (gift of Dr R. van Lier, Central Laboratory of the Blood Transfusion Service of the Netherlands Red Cross, Amsterdam, Netherlands). The labeled cells were removed by using magnetic beads coated with sheep anti-mouse immunoglobulins (DynaIc, Oslo, Norway) and a saranium cobalt magnet. The remaining cells after the first depletion were labeled with anti-CD34 (L185, from Dr J.H. Phillips), anti-CD19 and anti-CD14 (CLB CD19 and CLB CD14, respectively, from Dr R. van Lier) to remove the NK, B, and myeloid cells and again were subjected to depletion with magnetic beads. The enriched cells were incubated with anti-CD34 fluorescein isothiocyanate (FITC) and anti-CD38 PE (HPCA-2 and Leu 17, respectively, both from Becton Dickinson, San José, CA). CD34+CD38+ and CD34+CD38- cells were sorted with a FACStar plus (Becton Dickinson).

Fetal liver cells were homogenized through a wire mesh. After density gradient centrifugation over Ficoll (Lymphoprep; Nycomed, Minneapolis, MN) and anti-CD38 PE (HPCA-2 and Leu 17, respectively, both from Becton Dickinson, San José, CA), CD34+CD38+ and CD34+CD38- cells were sorted with a FACStar plus (Becton Dickinson).

Fetal liver cells were homogenized through a wire mesh. After density gradient centrifugation over Ficoll (Lymphoprep; Nycomed Pharma, Oslo, Norway), single cell suspensions were incubated with antibodies against glycophorin A (10F7 MN, obtained from the American Type Culture Collection, ATCC, Rockville, MD) and against the lineage markers CD4 (RPA-T4), CD8 (RPA-T8), CD5 (L 185), CD19 (CLB CD19), and CD14 (CLB CD14) (The CLB monoclonal antibodies [MoAbs] were kindly provided by Dr R. van Lier). The labeled cells were removed by two rounds of depletion with magnetic beads. Remaining cells were labeled with anti-CD34 and anti-CD38 and MoAbs and subjected to sorting on the FACStar plus. Only cell populations that were >99% pure were used in our studies.

**Hybrid human/mouse fetal thymic organ cultures.** The in vitro development of human T and NK cells from CD34+ fetal liver cells or thymocytes was studied using the hybrid human/mouse fetal thymic organ culture (FTOC). Fetal thymuses were obtained from embryos of C57Black 6 or RAG-1- deficient mice on, respectively, day 14 and day 15 to 16 of gestation and precultured for 5 days in the presence of 1.35 mmol/L 2-deoxyguanosine to remove endogenous thymocytes. The results obtained with the thymi of these two strains were qualitatively fully comparable (Res P, unpublished observations), but the RAG-I thymi could be used at day 15 to 16, whereas the use of Black 6 thymi was restricted to the gestational age of day 14, making routine use of the RAG-I thymi more easy.

Next, the thymic lobes were cocultured for 2 days in hanging drops in Terasaki wells with fluorescence-activated cell sorter (FACS)-sorted human progenitor cells, transferred to nuclease filters that were layered over gelfoam rafts in 6-well plates and cultured for the indicated number of days. Culture medium consisted of Yssel’s medium supplemented with 2% normal human serum and 5% fetal calf serum. To analyze differentiation of human cells, the mouse thymuses were dispersed into single-cell suspensions and stained with MoAbs specific for human cell surface antigens.

**Development of dendritic cells.** Development of CD34+ fetal liver cells and thymocytes to dendritic cells was studied in an in vitro culture system described by Caix et al. Ten thousand progenitor cells were cultured in Yssel’s medium supplemented with 2% normal human serum in the presence of 10 ng/mL stem cell factor (SCF; R&D Systems, Minneapolis, MN) of 50 ng/mL of granulocyte/macrophage colony-stimulating factor (GM-CSF; a kind gift of Dr R. Kastelein, DNAx) and 50 U/mL tumor necrosis factor (TNF) α (R&D) in 96-well microtiter plates (Costar). Appearance of dendritic cells was monitored by inspecting the morphology and analyzing the phenotype by flow cytometry. Limiting dilution cultures, to determine the frequency of the progenitor cells, were set up in round-bottomed 96-well plates. To this end we added 100 μL of a suspension containing CD34+CD38-CD50+ cells (at a concentration of 10 cells/mL) to each well followed by incubation for 2 to 3 weeks in culture medium with 10 ng/mL SCF, 50 ng/mL GM-CSF, and 50 U/mL TNFα. In one experiment single cells were sorted in microwells using the FACStar plus.

**Development of NK cells.** Sánchez et al. have demonstrated that CD34+CD5+ progenitors can develop into NK cells on a feeder layer of melanoma cells, SCF (R&D), IL-7 (R&D), and IL-2 (Eurocetus, Amsterdam, The Netherlands). In a pilot experiment we observed a strong growth of thymic NK progenitor cells when we used freshly isolated thymic stromal cells as feeder cells in addition to a combination of SCF, IL-7, and IL-2. To isolate fresh thymic stromal cells we took advantage of our finding that all hematopoietic cells in the thymus, including the CD34+CD38+ cells, express high levels of CD50 (ICAM-3, a ligand of CD11a/CD18), whereas the CD34+CD38- cells are invariably CD50 negative (E. Martinez Cáceres et al, manuscript submitted). A suspension of fetal thymocytes was prepared, and CD4+ and CD8+ cells were removed by magnetic bead sorting. The remaining cells were labeled with anti-CD34-FITC, anti-CD38-PE, and anti-CD50 biotin-StreptavidinCyCr (anti-CD50 MoAb was a kind gift of Dr Vilella, Barcelona, Spain) after which we sorted the CD50- and the CD34+CD38-CD50- cells. Three hundred CD50- cells were seeded per well of round-bottomed microtiter plates (Costar). Then we added 100 μL of a suspension containing CD34+CD38+CD50- cells (at a concentration of 10 cells/mL) to each well. In one experiment we sorted single cells in microwells using the FACStar plus. The cells were cultured for 2 to 3 weeks in Yssel’s medium with 2% normal human serum in the presence of 10 ng/mL SCF and 10 ng/mL IL-7 and 100 U/mL IL-2. We included as controls cultures of sorted CD50+ stromal cells and cytokines without CD34+CD38- cells.

**FACS analysis.** FACS analysis MoAbs against the following cell markers were used: CD3 (anti-Leu4), CD4 (anti-Leu3a), CD8 (anti-Leu2a), and CD56 (anti-Leu 19) from Becton Dickinson (San Jose, CA); CD45RA (anti-CD45RA PE, a gift of Dr van Lier and anti-CD45RA FITC from Becton Dickinson); and anti-Thy-1 (5E3, a generous gift of Dr P. Lansdop, Terry Fox, Vancouver, Canada). Anti-CD5 and anti-CD7 Triclor (TRC)-labeled antibodies were purchased from Caltag (San Fransisco, CA). Immunofluorescence staining to determine the cell surface phenotype was carried out as follows: 2 × 10⁴ cells were stained for 30 minutes on ice with the indicated directly labeled MoAbs, and after two washings were stained with Streptavidin-Cy Chrome (CyCr) (Caltag), whenever biotinylated MoAbs were used in the first step. After another two washings, cells were subjected to flow cytometric analysis using a FACSScan (Becton Dickinson). Fetal liver cells were routinely preincubated for 15 minutes with normal mouse serum IgG to avoid nonspecific binding of the MoAbs.

**RESULTS**

The cell surface phenotype of CD34+CD38- thymocytes. We found previously that the human fetal thymus contains a minute population of CD34+CD38- cells (<0.01% of the total number of thymocytes). These cells did not express the lineage markers CD2 and CD5, raising the possibility that these cells represented pluripotent stem cells since CD34+CD38- lineages cells in the bone marrow, fetal liver, and neonatal cord blood include pluripotent stem cells. Craig et al. have shown that the CD34+CD38- fetal liver cell population shows a gradient in the expression of Thy-1 from positive to negative and that all Thy-1- cells within this population lack the expression of CD45RA. Appearance of CD45RA is
correlated with disappearance of Thy-1. We could confirm this phenotype for fetal liver cells with the reagents we use (results not shown). The expression profile of Thy-1 on fetal CD34+ thymocytes is, however, different from that of the fetal liver cells (Fig 1A). While many CD34+CD38dim fetal liver cells express intermediate levels of Thy-1,13 almost no CD34+CD38dimThy-1int cells were detected in thymocyte samples (Fig 1A). An extremely small number of cells may be present that express intermediate levels of Thy-1, but it cannot be ruled out that their Thy-1 expression results from aspecific staining. Two distinct populations of CD34+CD38dim cells were found in fetal thymocytes. One negative for Thy-1 and another expressing very high levels of Thy-1. However, in contrast to the CD34highThy-1− cells the vast majority of CD34highThy-1high cells lack expression of CD45 and are presumably not hematopoietic cells (Fig 1B). This notion is substantiated by the finding that in contrast to CD34+Thy-1− thymocytes, purified CD34+Thy-1+ cells do not respond to hematopoietic factors such as SCF, GM-CSF, TNFα, or IL-7 but grow as adherent cells in media containing epidermal growth factor (E. Martinez Cáceres et al, manuscript in preparation).

Analysis of expression of CD45RA on CD34+CD38dim fetal thymocytes, depleted of Thy-1high cells, revealed that the cells with the highest CD34 expression are all positive for CD45RA, while some of these cells have low levels of CD38 (Fig 1C). Fig 1C, which shows a three-color analysis of a fetal thymic sample using anti-CD45RA FITC, anti-CD38 PE, and anti-CD34 biotin-streptavidin CyCr, demonstrates that all CD34highCD38dim cells express CD45RA, whereas about half of the

Fig 1. Phenotype of CD34+ thymocytes. (A) Expression of Thy-1 on CD34+CD38dim fetal thymic cells. Fetal thymocytes were depleted of CD4+ and CD8+ cells and stained with anti-CD34-FITC, anti-CD38-PE, and anti-Thy-1-biotin/CyCr.
CD34<sup>dim</sup>CD38<sup>+</sup> cells do not express CD45RA. Figure 1D shows more clearly that CD45RA expression decreases when cells lose the expression of CD34. This latter result is consistent with the fact that thymic progenitors lose CD45RA and acquire CD45RO upon further differentiation. These results indicate that the CD34<sup>+</sup>CD38<sup>dim</sup> cells in the thymus express CD45RA but no Thy-1 and are therefore phenotypically distinct from pluripotent stem cells.
CD34+CD38dim thymocytes develop into T cells in a human/mouse FTOC. The capacity of CD34+CD38dim thymocytes to develop into T cells was tested in a human/mouse hybrid FTOC. To avoid inclusion of the Thy-1dim CD34+ stromal cells in our preparation, we labeled the cells with anti-CD34-FTTC, anti-CD38-PE, and anti-Thy-1-CyCr and sorted the CD34+CD38dim Thy-1-negative cells. In a representative experiment shown in Fig 2, we purified 10,000 CD34+CD38dimThy-1- cells (which were >99% pure upon reanalysis) starting from 5 x 10^6 fetal thymocytes of a gestational age of 16 weeks. The purified CD34+CD38dim thymic cells developed into T (Fig 2A) and NK cells (Fig 2B) in 3 weeks of incubation after introduction in the mouse thymic organs. The CD34+CD38+ cells from the thymus also developed into T and NK cells. The proportion of NK cells developed from CD34+CD38+ thymocytes in the FTOC (0.2%, Fig 2B) was considerably lower than the percentage of NK cells developed from the CD34+CD38dim thymic cells (2%, Fig 2B). These results demonstrate that both the CD34+CD38dim and the CD34+CD38+ thymic cells contain progenitor cells with T and NK developing potential, but no conclusions with regard to the relative numbers of NK progenitors in each population can be inferred from these experiments.

CD34+CD38dim thymocytes are able to develop into dendritic cells. To test whether human CD34+CD38dimThy-1- cells can develop into dendritic cells, we cultured these cells in a combination of GM-CSF and TNFα, since Caux et al have demonstrated that these cytokines induce development of dendritic cells from CD34- cord blood cells. SCF was added because this cytokine increases the effect of GM-CSF and TNFα. In a representative experiment shown in Fig 3, we observed a 10-fold increase of cell number of the CD34+CD38dim cells after 7 days of culture compared with a twofold reduction of the number of cells in the CD34+CD38+ population. In two other experiments we observed 15- and 25-fold differences in cell recovery between CD34+CD38dim and CD34+CD38+ cells cultured in GM-CSF, SCF, and TNFα. Figure 3 shows that the great majority of the cells express CD14, CD40, and high levels of HLA-DR, while a proportion of these cells express CD1. Although not shown these cells were also negative for CD3, CD56, CD8, CD10, CD14, and CD19. In addition, these cells had a clear dendritic-like morphology (Fig 4). The recovery of CD34+CD38+ cells cultured for 1 week in SCF, GM-CSF, and TNFα was much lower, but the majority of cells expressed the same antigens as the cultured CD34+CD38dim cells. This result suggests that the frequency of dendritic progenitors in the CD34+CD38dim cells is higher than that in the CD34+CD38+ population.

The frequency of NK and dendritic cell progenitors in CD34+CD38dim thymocytes. To test the frequency of dendritic progenitors, we performed a limiting dilution assay of the CD34+CD38dim and CD34+CD38+ cells and inspected the microwells after 3 weeks of culture in SCF, GM-CSF, and TNFα. Positive cultures contained 10 to 50 cells and showed a typical dendritic appearance. Combined with the finding that bulk cultures of CD34+CD38dim cells in GM-CSF, SCF, and TNFα show a rather homogeneous dendritic morphology and phenotype, we can assume that the cells observed in the limiting dilution cultures are dendritic cells. Table 1 demonstrates that in exp 1, 2, 3, 5, and 6 the proportions of wells seeded with one CD34+CD38dim fetal thymus cell that contained growing dendritic cells ranged between 28% and 53%. The proportions of dendritic cell precursors in the CD34+CD38+ population as determined in exp 1 and 2 were about 20-fold lower, consistent with the idea that the majority of the cells in this population is already committed to the T-cell lineage. These data conclusively demonstrate the presence of dendritic progenitor cells in the CD34+CD38dim thymic cell population.

Sánchez et al reported that close to 100% of CD34+CD5 thymocytes (which include the CD34+CD38dim cells) can develop into CD56+ NK cells on a feeder layer of adherent cells in the presence of SCF, IL-7, and IL-2.11 To confirm that CD34+CD38dim thymocytes can develop into NK cells, we cocultured individual CD34+CD38dimCD50+ cells together with fresh CD50- thymic stromal cells and cytokines for 14 days. Table 1 shows that in three experiments (exp 4, 5, and 6) the proportions of wells with NK cells growing from single CD34+CD38dimCD50+ cells were 50%, 82%, and 48%, respectively. FACS analysis of clones expanded for 3 weeks in SCF, IL-2, and IL-7, after staining with anti-CD56-FITC revealed that always >90% of the cells were CD56- (data not shown). By contrast, the wells seeded with stromal cells only and cultured with the cytokine mixture did contain only adherent cells and no CD56+ cells could be found. Most interesting, in exp 5 we found that the frequencies of dendritic and NK cell progenitors in the same CD34+CD38dim cell sample were 53% (9 of 17) and 82% (14 of 17) indicating that a substantial proportion of these cells were bi-potential dendritic/NK progenitors. Since the frequencies as determined by limiting dilution may not be entirely accurate, we repeated the exp 5 but now seeded the wells by single cell sorting with a FACstar (exp 6). The observed frequencies of dendritic and NK cell progenitors in this experiment were 36% and 48%, respectively. As the frequencies deduced from the in vitro experiments are an underestimation, this result is consistent with the notion that the CD34+CD38dim thymocytes contain cells with at least 2 bipotential differentiating ability.

CD34+CD38dim but not CD34+CD38+ fetal liver cells develop into T and NK cells in a hybrid mouse/human FTOC. The finding that CD34+CD38dim thymic cells contain progenitors for T, NK, and dendritic cells suggests that these cells include the progenitor cells that enter the thymus. If this is correct, we would expect that CD34+CD38dim cells from fetal liver or bone marrow migrate to the thymus and enter the thymus without upregulating CD38. This hypothesis would be supported if LTC34+CD38+ fetal liver cells do not contain T-cell progenitors. To test this, we sorted CD34+CD38dim and CD34+CD38+ cells from fetal liver and tested the capacity of these two cell populations to differentiate into T cells in the FTOC. We found that CD34+CD38dim fetal liver cells develop into T cells, whereas in contrast to the thymic CD34+CD38+ cells, CD34+CD38- cells from the
Fig 2. The capacity of CD34⁺CD38⁺⁺ and CD34⁺CD38⁺⁻ thymic cells to differentiate into T and NK cells. Thymocytes were depleted of CD4⁺ and CD8⁺ cells as indicated in Materials and Methods and were sorted into CD34⁺CD38⁺⁺ and CD34⁺CD38⁺⁻ cells using the gates indicated in Fig 2A. The sorted cells were cultured for 3 weeks in mouse thymic organs, homogenized, and analyzed for expression of T- and NK-cell markers. (A) Staining with anti-CD3, -CD4, and -CD8 MoAbs. (B) Staining of the cells harvested from the FTOC, with anti-CD56 and anti-TCR γδ MoAbs. One other experiment yielded similar results.
fetal liver are unable to develop into T cells (Fig 5). In fact, almost no human cells could be recovered from the thymic organs cultured with CD34⁺CD38⁺ progenitor cells, precluding a phenotypic analysis of cells developed from CD34⁺CD38⁺ fetal liver cells. The inability of the CD34⁺CD38⁺ fetal liver cells to give rise to T cells in the FTOC was found in three out of three experiments. In one of these three experiments we assayed a small range of different cell numbers of both CD34⁺CD38dim and CD34⁺CD38⁺ fetal liver cells. The most important observation was that 6,000 human cells were harvested per fetal thymic lobe starting with down to 750 CD34⁺CD38dim cells, whereas no human cells were obtained from lobes grafted with 6,000 CD34⁺CD38⁺ fetal liver cells per lobe. In the other two experiments the same result was obtained starting from 5,000 CD34⁺CD38⁺ fetal liver cells per lobe. This lack of development in the FTOC was not due to lack of hematopoietic function; we have found that CD34⁺CD38⁺ fetal liver cells can differentiate to NK cells in the presence of SCF, IL-7, and IL-2, into monocytes in the presence of stromal cells, and into dendritic cells in the presence of SCF, GM-CSF, and TNFα (data not shown). In addition, the lack of T-cell potentiality of the CD34⁺CD38⁺ fetal liver cells is not due to the presence of cells within the CD34⁺ population, that suppress T-cell development because unfraccionated CD34⁺ cells, including both CD38dim and CD38⁺ cells, develop into T cells in a way comparable to CD34⁺CD38dim cells (data not shown).

**DISCUSSION**

We have shown previously that the human fetal thymus is capable to support not only development of T cells but also of NK, B, monocytes, and dendritic cells. We also found that the thymic microenvironment is able to support myelopoiesis in vitro, although much less efficiently than a combination of the cytokines, GM-CSF, SCF, IL-3, and IL-6. Moreover, we demonstrated that the human fetal thymus contains a minute CD34⁺CD38dim cell population that is negative for the T/NK markers CD2 and CD3. Those findings have led us to hypothesize that uncommitted CD34⁺CD38dim cells from fetal liver or the bone marrow seed the fetal thymus and have the ability to develop into T cells, but can also give rise to other hematopoietic lineages in that organ.

The results in this report support this hypothesis. First, we demonstrate that CD34⁺CD38dim thymocytes can differentiate into dendritic-like cells confirms the observations of Mirquez et al and extend them by further specifying these dendritic progenitor cells. Although our observations suggest that individual cells in the CD34⁺CD38dim thymocyte population are multipotent. Furthermore, we demonstrate that the T-cell potentiality of the fetal liver CD34⁺ cells is restricted to those cells that express no or only very low levels of CD38, indicating that CD34⁺CD38⁺ fetal liver cells do not contain pro-T cells. Our data do not preclude that more differentiated CD34⁺CD38⁺ fetal liver cells migrate into the thymus, but if this is the case, these cells do not differentiate to T cells after arrival in the thymus.

The finding that CD34⁺CD38dim thymocytes can differentiate into dendritic-like cells confirms the observations of Sánchez et al and extend them by further specifying these dendritic progenitor cells. Although our observations suggest that individual cells in the CD34⁺CD38dim thymocyte population have the capacity to develop into at least three cell types, it should be noted that this is not rigorously proven because a clonal analysis of the T-cell–differentiating capacity of the CD34⁺CD38dim thymocyte progenitors cannot be performed. However, Sánchez et al have shown that there are bi-potential T/NK progenitors in the CD34⁺CD5⁺ population (that includes the CD34⁺CD38dim cells), and when one accepts the premise that the branching of NK and T cells occurs downstream of the divergence of dendritic cells, a significant proportion of the CD34⁺CD38dim cells should be tripotential.
MULTIPOTENT CD34+ PROGENITORS IN HUMAN THYMUS

These cells are, however, distinct from pluripotent stem cells, because, in contrast to CD34+CD38dim cells from bone marrow, fetal liver, and neonatal cord blood, thymic CD34+CD38dim cells express CD45RA and lack Thy-1. In addition to two distinct populations of Thy-1- and Thy-1high cells among the CD34+CD38dim thymocytes, an extremely small population of Thy-1high cells was occasionally observed (Fig 1A). These CD34+ thymic cells could be real if the dots in the CD34+Thy-1dim region of the dot plot in Fig 1A are not an artifact of the staining. A functional analysis of these cells would however be severely hampered by the extremely small number of these cells in the fetal thymus. Thus, at present we cannot conclusively rule out that the thymus contains stem cells that are more immature than the CD34+CD38dimThy-1- cells.

The results reported here have implications for the question whether progenitor cells are committed to the T-cell lineage when they enter the thymus. Several groups have characterized candidate-committed T-cell progenitors in the fetal liver,21-24 giving credence to the notion that T-cell commitment occurs in the fetal liver before migration of progenitors to the thymus. However, the results of these studies are not conclusive, mainly because none of the markers used to define T-cell commitment, such as CD2, cytoplasmic CD3, CD5, and CD7, distinguish between early T- and NK-cell precursors.25 In a recent study we reexamined the question whether the expression of CD7 on CD34+ fetal liver cells would define T-cell commitment.26 Although CD34+CD7+ cells can indeed develop into T cells in a human fetal thymic organ culture, this population also contains progenitors of

Table 1. Frequencies of Dendritic and NK Cell Progenitors in CD34+ Thymocytes

<table>
<thead>
<tr>
<th>Fetal Thymus</th>
<th>Cytokines</th>
<th>No. of Positive Wells</th>
<th>CD34+CD38dim</th>
<th>CD34+CD38+</th>
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<tr>
<td></td>
<td></td>
<td>1 Cell/Well</td>
<td>10 Cells/Well</td>
<td>1 Cell/Well</td>
</tr>
<tr>
<td>1</td>
<td>SCF, GM-CSF, TNFα</td>
<td>12/36 (33)</td>
<td>20/20</td>
<td>0/36</td>
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<tr>
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<td>16/36 (44)</td>
<td>10/10</td>
<td>1/36 (3)</td>
</tr>
<tr>
<td>3</td>
<td>SCF, GM-CSF, TNFα</td>
<td>7/25 (28)</td>
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<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>SCF, IL-7, IL-2 stromal cells*</td>
<td>8/32 (50)†</td>
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</tr>
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<tr>
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<td>9/17 (53)</td>
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<tr>
<td></td>
<td>SCF, GM-CSF, TNFα</td>
<td>12/33 (36)</td>
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</table>

NK clones were inspected by FACS analysis after 2 to 3 weeks and were >90% CD56-. Dendritic clones were small (50 to 100 cells) but had distinct dendritic morphology. Values in parentheses are percents.

* Stromal cells alone did not give rise to either NK or DC with the indicated cytokines.
† In this experiment 1 cell per two wells was seeded.
CD4+CD38− cells are not committed to the T-cell lineage. The results reported here strongly suggest that the fetal liver does not contain committed T-cell progenitors at all. If these cells were present, they would be expected to be included in the CD34+CD38− fetal liver cell population, because differentiation of stem cells is accompanied by upregulation of CD38. However, CD34+CD38− fetal liver cells are unable to differentiate into T cells. The results with fetal liver progenitors support the notion that primitive hematopoietic progenitors leave the fetal liver to migrate to the thymus without being committed to the T-cell lineage in the liver.

In this report we examined the fetal liver and fetal thymus within a window of gestational age (15 to 16 weeks) in which the thymus is presumably populated with progenitor cells from the fetal liver. Galy et al reported recently that CD34+Thy-1−CD45RA−CD10+ cells from fetal and adult bone marrow had lymphoid- and dendritic-cell-developing potential. The great majority of these cells express CD38. These cells have lost the ability to develop into cells of the erythroid and myeloid lineage. Except for CD38 these cells resemble the early fetal thymic progenitors that we have characterized in this study. It may be possible that CD34+CD38+Thy-1− CD45RA− cells in the bone marrow convert to CD34+CD38−Thy-1−CD45RA+ cells before migrating to the thymus. We have found that CD34+CD38−CD45RA+ cells are also present in the postnatal thymus and can develop into T cells in the human/mouse FTOC (results not shown), suggesting that CD38 is not yet fully upregulated when the early progenitors arrive in the thymus after birth. However, at present we cannot exclude that a portion of CD34+ cells that enter the thymus after birth express CD38. On the basis of the premise that thymic progenitors are blood-borne, we are at present analyzing the T-cell-developing potential of CD34+CD38− and CD34+CD38− neonatal cord blood progenitors to settle the phenotype of the CD34+ cells that migrate to the thymus at birth.

The notion that progenitor cells that enter the thymus are not yet committed to the T-cell lineage is supported by data from studies in the mouse. The most immature adult thymocytes express Sca-1 (Ly-6A), Sca-2, c-kit, and low levels of Thy-1 and CD4. The expression profile of immature thymocytes resembles that of a cell population enriched for pluripotent stem cells but is yet distinct because in contrast to stem cells the CD4− thymocytes express Sca-2. These early thymic precursors are capable to develop into T, NK, and dendritic cells upon intrathymic transfer and to B cells after intravenous injection. Although there may be differences between mouse and human with respect to the earliest thymic progenitors, our findings may be extrapolated to the mouse in that single progenitors in the early thymic progenitor pool can develop into T, NK, and dendritic cells. Surprisingly, mouse CD4+ cells from fetal thymus were not able to develop into T cells, suggesting that the CD4+ thymic progenitor cells represent an early stage in T-cell development in adult but not in fetal mouse thymus. This difference between fetal and adult mouse thymic CD4+ progenitors contrasts with the presence of functional CD34+CD38−CD45RA+ progenitors in both fetal and in postnatal human thymus.

Although it is clear that the mouse thymus contains uncommitted hematopoietic progenitors, findings in one study...
suggest that at least some early prothymic progenitors in the mouse are committed to the T-cell lineage before they enter the thymus. These authors analyzed the T-cell—developing capacity of progenitors in peripheral blood of embryos at 15.5 days of gestation. At that time the thymus is colonized, but the cells in the thymus have not progressed beyond the CD4−CD8+ stage. A population of cells was identified which express Thy-1 and low levels of c-kit. These cells could develop into T cells, but not to B or myeloid cells. A small percentage of Thy-1+c-kitdim fetal blood cells had undergone D/β to Jβ rearrangements, indicating the presence of committed T-cell progenitors in this population. If the fetal peripheral blood Thy-1+c-kitdim cells are thymic precursors, they are only populating the thymus during fetal development, as these cells are undetectable in the blood of new born mice. It cannot be excluded that the Thy-1+c-kitdim cells found in the study of Rodewald et al represent cells that migrated back from the thymic rudiment to the periphery. It might be possible that at day 15.5 when the medulla is not yet formed, cells can migrate out of the thymus before completion of differentiation.

Taking together the results of studies in mouse and in human, we favor the interpretation that the thymus is entered by a multi-potent progenitor cell distinct from stem cells with the capacity to develop into T, NK, and dendritic cells and probably also to myeloid cells. The human thymic microenvironment is permissive for development of non-T-cell lineages. It is therefore feasible that some of the multi-potential progenitors entering the thymus develop into cells of the NK, B, or myeloid lineages in that organ.

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CD34+CD38dim cells in the human thymus can differentiate into T, natural killer, and dendritic cells but are distinct from pluripotent stem cells

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