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In Vitro Intrathymic Differentiation Kinetics of Human Fetal Liver CD34+CD38− Progenitors Reveals a Phenotypically Defined Dendritic/T-NK Precursor Split

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Human CD34+CD38− hematopoietic precursor cells from fetal liver are able to develop into T, NK, and dendritic cells in a hybrid human/mouse fetal thymic organ culture (FTOC). In this report, we pay particular attention to the early events in differentiation of these precursor cells. We show that the CD34+CD38− precursor cells, which are CD4−CD7−cyCD3−HLA-DR−/++ (cy, cytoplasmatic), differentiate into a CD4+ population that remained CD7−cyCD3−HLA-DR−/+ and a CD4+ population that expressed CD7 and cyCD3. The CD4+CD7−cyCD3− cells differentiate into phenotypically and functionally mature dendritic cells, but do not differentiate into T or NK cells. The CD4−CD7+cyCD3+ population later differentiates into a CD4−CD7+cyCD3−HLA-DR+ population, which has no potential to differentiate into dendritic cells but is able to differentiate into NK cells and γδ and αβ T lymphocytes. These findings support the notion that the T/NK split occurs downstream of the NK/dendritic split. The Journal of Immunology, 1999, 162: 60–68.

The thymus is the major site of T lymphocyte differentiation. Besides T cells, dendritic cells and NK cells can complete their differentiation pathway in the thymus microenvironment. Much of the knowledge of the molecular processes and genes involved in thymus differentiation has come from studying transgenic and gene knock-out mice (for review see Ref. 1). For obvious reasons, these studies are not applicable in humans. However, other approaches are possible. Studies of intrathymic developmental pathways in the mouse relying on the use of an in vitro fetal thymus organ culture (FTOC) system (2, 3) and intrathyMIC injection of putative progenitor cells into host mice have defined the differentiation potential of different subsets of thymocytes. Highly purified candidate progenitor subsets are assayed by adoptive transfer. The lineages that appear and the time course of appearance of more mature subsets of thymocytes, such as CD4+CD8+ (double positive, DP) or TCR-αβ high, CD4+CD8-, or CD4-CD8- (single positive, SP) cells, serve as indicators of the degree of maturation of the donor population. Using these techniques, a very small population of thymocytes has been identified that seems to include early progenitors. In mice, these cells are identified by the expression of CD44, high levels of class I MHC, low levels of CD4 and heat stable Ag, and absence of CD3 and CD8 (4, 5). In adoptive transfer experiments, these cells give rise to B cells as well as T cells, but not to myeloid cells.

The study of intrathymic developmental pathways in humans relies on phenotypic analysis. Particular seminal has been the discovery of CD34 as a stage-specific marker identifying immature hematopoietic stem cells (6, 7). The expression level of CD34 and its coexpression with differentiation markers as well as the capacity of these subsets to differentiate in FTOC allowed the delineation of different stages in human intrathymic development (8). More recently, on the basis of their ability to reconstitute thymopoiesis in ectopic human fetal thymus implants in immunodeficient C.B17 scid/scid (SCID) mice, the early stages of lymphoid cell formation of phenotypically defined subsets of CD34+ bone marrow cells have been defined (9). However, although bone marrow lymphoid precursors have been carefully studied, little is known about the sequential appearance of phenotypic markers during the first 2 wk after the entrance of the human precursor cell in the thymus. Phenotypic changes during the differentiation process have been proposed on the basis of multiparameter flow cytometric studies of the thymus (7), or more recently by assessing the differentiation of sorted thymus subpopulations in human FTOC (8, 10) or in murine FTOC (11), but data on the kinetics of the different lineages are lacking. Using a hybrid human/scid mouse FTOC system (12), we address in the present study the kinetics of the very early steps of differentiation of CD34+CD38−Lin− precursor cells derived from fetal liver by analysis of the sequential appearance of surface Ags and the expression of recombination activating gene 1 (RAG-1) and pre-TCR-α-chain (pTα). We report the existence of different phenotypic intermediates that produce lymphocytes, NK cells, and dendritic cells and demonstrate that the kinetics of these distinct pathways differ.

Materials and Methods

Animals

CB-17 SCID mice were obtained from our own specific pathogen-free breeding facility. For timed pregnancies, females were housed in separate
cages from the males until mating. The appearance of vaginal plug after overnight mating was labeled as day 0 of pregnancy. The 14- to 15-day pregnant mice were sacrificed to obtain the embryos for preparation of the thymic lobes.

**Abs and reagents**

The mAbs used were rat anti-mouse CD45 cytochrome (CD45, 30F1 1:1; PharMingen, San Diego, CA), biotinylated rat anti-mouse H-2 class I MHC H-2Kd (PharMingen), and the following mouse mAbs: anti-human glycophorin-A (10F7 MN; a kind gift of Dr. L. L. Lanier, DNAX, Palo Alto, CA), CD1a (B-B5; Serotec, Oxford, U.K.), CD38 (OKT10 FITC; Ortho, Raritan, NJ), CD2 (Leu-5b FITC), CD3 (anti CD3ε, Leu-4 FITC, phycoerythrin (PE)), CD4 (Leu-3a FITC or PE), CD7 (Leu-9 FITC), CD8 (Leu2a FITC), CD14 (LeuM3 PE), CD16 (Leu-11a FITC), CD19 (Leu-12 PE), CD20 (B-225 PE), CD34 (HPCA-2 FITC or PE), CD45 (HL-1 FITC), CD56 (clone NC16A PE), CD69 (clone H120 FITC), and anti-TCR-γ/δ FITC and anti-TCR-γ/δ-6F8 FITC (all from Becton Dickinson Immunocytometry Systems, Mountain View, CA). Human recombinant IL-15 (CHO-derived) was kindly provided by Immunex (Seattle, WA).

**Preparation of human CD34+CD38−Lin− fetal liver cells**

Human fetal liver tissues were obtained and used following the guidelines of the medical ethical commission of the University Hospital of Ghent. Human fetal liver cells were isolated by gentle disruption of the tissue in complete medium (Isco’s modified Dulbecco’s medium/10% FCS, Life Technologies, Paisley, Scotland) followed by centrifugation over Lymphoprep (Nycomed Pharma, Oslo, Norway). Cells were washed and resuspended in 90% FCS/10% DMSO and frozen in liquid N2. After thawing, fetal liver cells were washed and labeled with glycophorin A, CD19, and FITC-labeled mAbs CD1, CD3, CD4, CD7, CD8, and CD38. Ab-labeled cells were depleted by immunomagnetic beads. For this purpose, the murine mAbs reconstituted in 0.5 ml cold PBS/2% FCS and mixed with 0.5 ml prewashed (to remove the preservative) sheep anti-mouse Ig-coated Dynabeads (Dynal AS, Oslo, Norway) to obtain a ratio of cells to Dynabeads of 1:5. After 30 min at 4°C, the suspension was diluted by carefully adding 5 ml PBS/2% FCS, and the rosettes of cells with Dynabeads were removed by placing the tube on a magnetic particle concentrator (Dynal AS). The supernatant that contained the unlabelled and weakly stained cells was moved and centrifuged (500 x g for 6 min). These cells were labeled with CD34-PE and CD34‘CD38’ Lin− sorted. The sorted cells were transferred to murine thymic lobes by the hanging drop method (13).

**FTOC**

Thymic lobes were prepared from fetal day 14–15 SCID mice. Hanging drops were prepared in Terasaki plates by adding in each well 25 μl of complete medium containing 10,000 or less sorted human Lin− cells, where the dead cells were gated out according to different scatter properties. In most cases, viable human cells were gated by exclusion of mouse CD45+, mouse class I MHC+ cells, and PI-positive cells.

**Allogeneic MLC assays**

PBL were obtained by centrifugation of blood from healthy adult volunteers over Lymphoprep (Nycomed Pharma). PBL were resuspended in RPMI 1640 with 10% filtered human AB serum and 5 x 10−3 M 2-ME (complete MLC medium). MLC were set up in 0.2 ml complete MLC medium in round-bottom 96-well trays (Falcon, Becton Dickinson, Lincoln Park, NJ). The stimulator cells were either sorted human thymocytes from FTOC after 11 days of culture (2 x 102 – 2 x 103) or sorted CD4+HLA-DR−, and further processed following the instructions of the supplier. Then, 10 μg of Escherichia coli tRNA (Boehringer, Indianapolis, IN) was added as a carrier before chloroform addition and cDNA was prepared using Superscript (Life Technologies) according to the specifications of the supplier. An aliquot of each cDNA sample was subsequently amplified in twofold serial dilutions by PCR using pTα, RAG-1, and HPRT-specific primers. In each PCR, water, SCID-mouse fetal thymus cDNA, and human and mouse genomic DNA were included as negative controls. All primer pairs amplified human cDNA only. PCR amplification using the different primers was performed in 50 μl volumes with 1 U of Taq polymerase (Perkin-Elmer, Emeryville, CA) using a 96-well thermocycler (Omnigene, Hybaid Teddington, U.K.) under the following conditions: pTα, 35 cycles of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C; RAG-1 and HPRT, 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The sequences of the primers were as follows: for pTα, 5’TGG AGG CCC ACC ACG TTG TGG’ (sense primer) and 5’–TGA ACG TCT CTC ATG GCA TCA T’ (anti-sense primer); for RAG-1, 5’TGA CCA AGT GCA GTC TCA T’ (sense primer) and 5’–CAT CAC CTA GCC TGG GAT ACA C’ (anti-sense primer); for HPRT, 5’–AAT TAT GGA CAC GAC TGA ACG T’ (sense primer) and 5’–TCA AAT CCA ACA AGA TCT GGC TTA A’ (anti-sense primer). HPRT was visualized on ethidium bromide stained gels. PCR products of pTα and RAG-1 were Southern blotted to Nytran paper (Schleicher & Schuell, Keene, NH). The membrane was hybridized at 60°C using a 32P-end-labeled internal oligonucleotide, for pTα 5’-CC TCT CCT GGC CCA CCA A TC A3’ and for RAG-1 5’-CC TCT CTG ACA TCT GCA A3’.

**Results**

**Detailed phenotypic analysis of early steps of differentiation**

We have already reported that during hanging drop approximately 1000 to 2000 progenitors cells, i.e., 1/5th to 1/10th of the input number of cells, enter the thymic lobe (12) and that afterward the number of cells, entering the thymic lobe (12) and that afterward the number of cells during the first days of the culture is low. When we electronically gated out the dead as well as the murine cells by eliminating the cells that stained with PI and anti-mouse CD45-Tri-Color, a population remained that did not stain for human CD45 (Fig. 1). When anti-mouse class I MHC I biotin-streptavidin-Tri-Color was added, we obtained a clearer picture as nearly all cells that were gated out electronically now stain for human CD45. Most likely, this CD45− population, which can be stained by anti-mouse class I MHC, represents a population of mouse epithelial cells. By gating out all...
nonhuman cells, we could carefully trace the human cells and study their phenotypic changes at the early stage of differentiation, even when the total cell number is very minute. Other systems, such as injection of mismatched HLA donor cells into transplanted SCID-human mice, do not offer the possibility to clearly gate out donor cells, because the Ag density of the HLA-marker is relatively weak during the first stages of differentiation (16). Therefore, the hybrid human/mouse FTOC system used in our study gives us a unique opportunity to address the earliest steps of differentiation.

Fig. 2A shows the cell surface expression of CD34, CD7, and CD38 and the cytoplasmic expression of CD3 (cyCD3) in function of duration of the culture. According the purification strategy, the sorted precursor cells were CD7- and CD38-negative (Fig. 2A) at the start of the culture. After three days of culture, most of the CD34+ cells already expressed CD7 and CD38, 70 and 85%, respectively. Very rapidly some cells coexpress CD34 cells with cyCD3 upon culture. However, an important fraction of the cells at that time has down-regulated CD34 and does not express cyCD3. After 4 to 5 days of culture, ~1000 to 2000 human cells can be found in one reconstituted thymic lobe. An important fraction of
the human cells expresses CD4 (30–50%, 300–900 cells/lobe) but does not express cyCD3 nor CD7 (Fig. 2B). At that time, cyCD3+ cells are CD4+CD7+ (Fig. 2B) and the expression of CD4 and CD7 is mutually exclusive (data not shown). After 7 days of culture, the number of human cells has increased to 3000–4000/lobe, part of the cyCD3+ cells starts to express CD4, but almost all the cyCD3+ cells remain CD7+. From this we conclude that there is a first wave of two cell populations that are either CD4+cyCD3+CD7+ or CD4+cyCD3+CD7− followed by the generation of a second wave of cells that are CD4+cyCD3+CD7+. As discussed below, further phenotypic analysis allowed us to delineate the differentiation of four different cell lineages: dendritic cells, NK cells, TCR-γδ, and TCR-αβ cells.

**Analysis of the development of dendritic cells**

After 3 days of culture, some human cells had down-regulated CD34 without cyCD3 expression (Fig. 2A). We analyzed whether these cells might represent dendritic cells. Because Sotzik et al. (17) have shown that human thymic dendritic cells are large cells expressing high levels of CD4 and class I and II MHC but no CD7, we analyzed these markers at different time points of FTOC. As seen in Fig. 3A, at day 4 the CD4+ cells express HLA-DR. Later, the intensity of both CD4 and HLA-DR increases, so that after 11 days a small but distinct population of HLA-DR+CD4+ cells can be discerned. The number of these cells is ~200–500 cells/lobe at day 4–6 and increases only two- to threefold until day 12; afterward, the number remains constant. As the total number of human thymocytes increases 10- to 20-fold (20,000–40,000 human cells/lobe), the frequency of these putative dendritic cells decreases in function of length of culture (Fig. 3A). After 12 days of FTOC, it is possible to define cells that highly express CD4, HLA-DR, and HLA-class I are negative for CD7 and cyCD3 (Fig. 3B) and have a high forward and side scatter (Fig. 3B, R6). We further corroborated that these cells were functional dendritic cells by assessing their ability to stimulate allogeneic T cells in an MLC. The maximal extent of proliferation of responder lymphocytes was similar to that seen with PBL as stimulators, but 80-fold fewer stimulator cells were required for peak stimulation (Fig. 3C).

To test at what stage of development the potential to generate dendritic cells is still retained, we tested the capacity of sorted human CD4+ HLA-DR− cells and CD4− cells from day 10 FTOC to develop into dendritic cells in a second human/mouse hybrid FTOC. In a representative experiment shown in Fig. 4, we purified these populations (which were >96% pure upon reanalysis). It is clear that dendritic cells and CD4+ precursor cells were generated starting from the CD4+ population, whereas the CD4+ HLA-DR+ population has lost its potential to develop into dendritic cells. Finally, the sorted CD4+ HLA-DR+ cells were end-stage cells that retained the dendritic phenotype and were not able to give rise to T cells (data not shown). These results demonstrate that the lymphoid/dendritic common precursor is CD4+, whereas the CD4+ (HLA-DR+) cell populations contain no dendritic precursors. Taken together, these results indicate that during differentiation, dendritic cells down-regulate CD34, express HLA-DR and CD4 and do not express cyCD3 and CD7.

**Analysis of the development of NK cells**

As only a small number of NK cells develop spontaneously in FTOC (Fig. 5), we took advantage of the property of IL-15 as a strong stimulator of NK cell development (18, 19) to study whether different thymic subsets were able to generate NK cells. Starting with CD34+Lin−CD38− cells, we were not able to generate NK cells with IL-15 in cell suspension (data not shown). However, when put into FTOC and cultured in the presence of...
IL-15, large numbers of CD3+CD56+ cells were generated. Fig. 5 shows that the addition of 20 ng/ml IL-15 to FTOC resulted in a dramatic increase in CD56+ cells. These cells had the appearance of large granular lymphocytes (data not shown). Further phenotypic analysis showed that part of the cells coexpresses CD16 (Fig. 5). In addition, we performed sorting experiments to obtain CD4+HLA-DR− and CD4− subsets from day 10 FTOC, and both subsets were able to generate NK cells when reintroduced in FTOC in the presence of IL-15 (Fig. 6). However, the starting CD34+CD38−Lin− population, as well as CD4+HLA-DR− cells from day 10 FTOC, were not able to generate NK cells in suspension cultures with IL-15. In contrast, CD4+ cells from day 10 FTOC generated NK cells when cultured in suspension in the presence of IL-15 (data not shown). This apparent paradox might be ascribed to a difference in sensitivity of the assays and that a small contamination of the CD4− cells in the CD4+ cells are able to generate NK cells in FTOC, whereas this does not occur in suspension. In this respect, the purity of the sorted cell populations was at least 96%, and the input of cells was 5000. Therefore, the absolute number of cells with the NK phenotype CD56+CD3− recovered per lobe, which is ~8,000–12,000 after 10 days of culture, does not rule out the possibility of outgrowth of contaminating cells. Alternatively, the fact that with IL-15 CD4+ cells can generate NK cells in FTOC and not in suspension, whereas CD4− cells generate NK cells in FOTC and in suspension, can be interpreted as the CD4− populations contain more mature NK precursor cells (day 10). Taken together, these results indicate that up-regulation of the CD4 marker on HLA-DR− precursor cells is accompanied with a loss of differentiation potential for dendritic cells, but that NK and T potential is still preserved. Therefore, these results suggest that the branching point of dendritic cells is earlier in the developmental scheme than the T/NK cell branching point.

Analysis of the development of T cells: purified CD34+CD38− fetal liver progenitor cells in FTOC and subsequently complete their differentiation

We have previously shown that 28 days of FTOC started with 10,000 CD34+ fetal liver cells resulted in the appearance of CD4+CD8−CD3+CD1+ human thymocytes (12). Here we show that after 40 days of culture CD1−TCR-αβ+ and CD1−TCRγδ+ thymocytes were generated by CD34+CD38− fetal liver cells and that the thymocytes had partially down-regulated CD45RO and acquired CD45RA (Fig. 7). At that time point of the culture, 50,000–150,000 human cells are found per lobe. In addition, part of the CD3+CD1−TCR-αβ+ cells were either CD4+CD8− or CD4−CD8+ (data not shown). We have shown previously that CD1−CD3−CD4+ or CD1−CD3−CD8+ cells have functional capacity and are considered as complete differentiated thymocytes (20). In addition, we have also demonstrated that CD1−TCR-γδ+ cells have functional characteristics of mature T cells (21).

Analysis of mRNA expression for RAG-1 and pTα

To assess the immaturity of the starting population, we analyzed the presence of RAG-1 and pTα mRNA transcripts. Expression of RAG genes is absolutely required for rearrangement of TCR genes, but is also present in developing B cells as it is required for the rearrangement of IgS. pTα is restricted to cells of the T cell lineage (22, 23). The expression of RAG-1 and pTα mRNA has already been addressed in the analysis of human thymic subsets (24). In Fig. 8, it is shown that pTα is already present in the CD34+CD38−Lin− starting population and that the expression is higher after 3 days of culture in FTOC. In contrast, although RAG-1 expression is sometimes weakly present in the starting population, it is not detectable in day +3 samples. RAG-1 mRNA expression could be clearly demonstrated in human thymocytes after 11 and 14 days of FTOC.
Discussion

In this study, we show that in a hybrid human/mouse FTOC, immature human stem cells from fetal liver spontaneously develop into at least four different lineages: dendritic cells, NK cells, TCR-γδ, and TCR-αβ T cells. This system allows a detailed kinetic analysis of the different cell populations, because a cohort of CD34^+CD38^- stem cells starts to develop resembling a synchronized culture. This is in contrast to analysis of different subsets in a human thymus, where all different cell populations are already present.

Moreover, in this study we are able to address the differentiation of pluripotent hematopoietic precursor cells from fetal liver, whereas most data on human T cell differentiation are founded on studies with postnatal thymus. However, the human/mouse FTOC method that we have used has potential shortcomings. First, we have introduced human pluripotent fetal liver cells with myeloid differentiation capacity (data not shown) in FTOC. This might not represent the default pathway, because it remains an open question whether an uncommitted pluripotent cell seeds the thymus or a more committed cell. Secondly, although the differentiation capacity of the fetal liver cells in the xenogeneic environment is impressive, we cannot exclude that critical factors or ligands that are species-specific are lacking and might influence the default differentiation pathway. Nevertheless, our experience with cord blood-derived hematopoietic precursors shows that the differentiation obtained in in vitro xenogeneic FTOC (25) is very similar to that obtained by precursor cells that were injected in vivo in human thymic tissue transplanted under the kidney capsule of SCID/human reconstituted mice (our unpublished observations). Therefore, we consider that our approach gives us an unique opportunity to study the T developmental pathway of prenatal fetal liver precursor cells. If the human/mouse FTOC recapitulates the normal differentiation of the human thymus, our results imply that immature CD34^+CD38^- human stem cells develop very rapidly into dendritic cells, that the differentiation toward NK cells takes somewhat longer in time, and, finally, that TCR-γδ cells are generated before TCR-αβ cells.

FIGURE 6. NK cell developmental potential of sorted CD4^+HLA-DR^- and CD4^-human cells from 10 day FTOC. Cell suspensions were prepared and the cells were stained with anti-CD4-PE and anti-HLA-DR-FITC and PI and anti-mouse CD45-cychrome and anti-mouse MHC class I MHC-biotin plus streptavidin-Tri-Color. This allowed us to gate out dead cells and mouse cells and select for CD4^+HLA-DR^- and CD4^- human cells. Each sorted population was cultured in a second FTOC in the presence of 20 ng/ml IL-15. After an incubation of 12 days, cell suspensions were prepared. Flow cytometric analysis was performed after staining the cells with mAbs against human CD2-PE and CD56-PE. It is obvious that both populations generated NK cells based on expression of CD56 and absence of CD2. This experiment is representative for two independent experiments. The percentages of cells within the quadrant of interest is indicated.

FIGURE 7. Flow cytometry analysis of human cells after 40 days of culture in hybrid human-mouse FTOC. Cultures were initiated with CD34^+CD38^- progenitor cells from fetal liver. After 40 days of culture, cell suspensions were prepared, and cells were stained with PI and anti-mouse CD45-cychrome and anti-mouse MHC class I MHC-biotin plus streptavidin-Tri-Color and PE or FITC directly conjugated mAbs as indicated. This allowed us to gate out dead cells and mouse cells and to analyze for the coordinate expression of CD4 vs CD8, CD4 vs CD3 (top panel), CD1 vs TCR-γδ, CD1 vs TCR-αβ, and CD45RA vs CD45RO (bottom panel) on human cells. Representative results of one of three experiments is shown.

FIGURE 8. Analysis of pTα and RAG-1 mRNA expression in fetal liver CD34^+CD38^- stem cells and in FTOC cells. Expression of mRNA was determined by semiquantitative RT-PCR analysis, in which the level of HPRT was used as a control for the amount of mRNA isolated. Five times more cDNA was used to determine RAG-1 and pTα compared with HPRT. HPRT is visualized on ethidium bromide stained gels, and PCR products of RAG-1 and pTα are visualized by Southern blots. The four bands on a gel represent a twofold dilution of one sample.
Dendritic cells comprise a family of professional APC responsible for the activation of primary T cell responses. Large numbers of functional dendritic/Langerhans cells can be generated by treating dendritic cell precursors from several sources with granulocyte-macrophage-CSF alone or in combination with other growth factors. More recently, it was also shown that ligation of CD40 induces the proliferation and differentiation of CD34 hematopoietic stem cells into dendritic cells (26). Sotzik et al. (17) have shown that dendritic cells in the human thymus can be identified as CD34<sup>high</sup>CD44<sup>+</sup>CD1<sup>-</sup>CD2<sup>-</sup>CD3<sup>-</sup>CD7<sup>-</sup>CD8<sup>-</sup>CD34<sup>-</sup> class I MHC<sup>high</sup> class II MHC<sup>high</sup> cells with efficient stimulator capacity for allogeneic T cells. We show here that dendritic cells are generated spontaneously within the thymus microenvironment from CD34<sup>-</sup>CD38<sup>-</sup> progenitor cells within 8-10 days. These cells are the earliest cells to be generated and to be functionally mature. The functional relevance of this population within the thymus remains to be established. There is no doubt from mouse studies that dendritic cells are essential for the negative selection of T cells, whereas epithelial cells mediate positive selection (27). However, at present it is not clear whether dendritic cells in the thymus originate from precursor cells that differentiate in the thymus or whether mature dendritic cells migrate from the periphery to the thymus. This is of interest because in the latter situation, Ags that are loaded in the periphery can participate in the negative selection process, whereas otherwise these Ags have to be produced locally or have to reach the thymus. Our findings show that dendritic cells are able to differentiate in the thymus from precursor cells and that the kinetics are faster than for the other cell types. This allows that negative selection can be mediated very rapidly when the T cells are at a very immature stage and supports the assumption that a linked development of T cells and dendritic cells may ensure that developing T cells are negatively selected predominantly by self-Ags presented on newly formed thymic dendritic cells. This idea was already proposed for the murine thymus, as it was shown that after irradiation bone marrow cells injected intrathymically restore simultaneously T cells and dendritic cells (28, 29).

Another cell type that develops spontaneously albeit in low numbers in human/mouse FTOC are NK cells (Fig. 5). It is obvious that most NK cells are generated outside the thymus as nude mice have a normal NK activity. However, the thymus provides a microenvironment that allows stem cells to differentiate toward the NK cell pathway. This is shown by the fact that the CD34<sup>+</sup>Lin<sup>-</sup> population from fetal liver did not differentiate toward NK cells in cell suspension when IL-15 was added, even in the presence of stem cell factor and IL-7 (data not shown), whereas in FTOC IL-15 resulted in a massive differentiation toward NK cells. Similarly, CD4<sup>+</sup> and CD4<sup>-</sup> precursor cells from day 10 FTOC also had the capacity to develop into NK cells in FTOC in presence of IL-15. However, only day 10 FTOC CD4<sup>-</sup> precursor cells, in contrast to the CD34<sup>-</sup>CD38<sup>-</sup> start population, were able to develop into NK cells in the presence of IL-15 in suspension cultures. This means that the stromal environment and/or cytokines present in the thymus can allow the differentiation of the IL-15 refractory CD34<sup>-</sup> precursor cell to a cell population that responds vigorously to IL-15 by cell proliferation and differentiation toward NK cells. It is clear from our studies that IL-15 is a potent promoter for the growth and differentiation of human NK cells. We also found that part of the NK cells, generated in IL-15-supplemented FTOC, coexpress CD16 (Fig. 5) and CD94 (data not shown). We have already shown previously the role of IL-15 as NK cell promoter in mouse fetal thymus (18). Our present observations point to an essential role of IL-15 in human NK development and are in agreement with the observation of Mingari et al. (30), who have been able to show that IL-15 provides an efficient stimulus for differentiation of thymic precursors toward phenotypically and functionally mature NK cells.

The precise role of NK development in the thymus remains unknown. Because the main physiological maturation of NK cells occurs in the bone marrow, it is possible that NK maturation in the thymus is not relevant. In particular, the fact that only CD94 is up-regulated and other HLA class-I specific inhibitory receptors are not expressed (data not shown, and Ref. 30) may support the hypothesis that this maturation is incomplete and not physiological.

The first T cells that develop in the mouse thymus are TCR-γδ cells. Indirect evidence that this is also the case for the human thymus is based on studies on T cell acute lymphoblastic leukemia. As CD3<sup>-</sup> T cell acute lymphoblastic leukemias have been found with rearranged TCR-δ genes and germline TCR-β and TCR-γ genes (31), it is probable that TCR-δ genes rearrange early during T cell development before other TCR genes. In addition, RT-PCR analysis performed by Ktorza et al. (32) showed expression of full-length TCR-δ transcripts in the CD34<sup>-</sup>CD1<sup>+</sup> TN thymocyte subset, before expression of full length TCR-β transcripts. In the earliest phase of fetal thymus colonization at 8.2 wk of gestation, by immunohistochemistry 5% of the thymocytes stained intracellularly with β-F1 mAb, suggesting expression of TCR-β-chain, whereas only a few TCR-γ<sup>-</sup> cells were found. The time of first appearance of TCR-δ is at 9.5 wk (33). In our study we do not have direct proof that differentiating thymocytes start earlier in their attempt to rearrange the TCR-γδ genes than TCR-αβ genes. We consequently found that cells with cell surface expression of TCR-γδ are present before we can detect T cells with TCR-αβ. However, as it is known that TCR-αβ cells first have to express TCR-β-chain with pTα before completion of the differentiation and expression of TCR-αβ, the difference in time of appearance of TCR-γδ and TCR-αβ cells might rather reflect a different kinetic in maturation of the two cell types.

Our finding that RAG-1 and pTα mRNA is present in the starting CD34<sup>-</sup>CD38<sup>-</sup> fetal liver cell population differs from the failure to detect expression of pTα and RAG-1 in the CD34<sup>-</sup>CD38<sup>-</sup> cell population of cord blood, as reported by Blom et al. (34), and in the CD34<sup>-</sup>CD38<sup>-</sup> population of fetal liver, as reported by Jaleco et al. (19). In contrast, Ramiro et al. were able to demonstrate pTα mRNA in CD34<sup>+</sup> progenitors in fetal liver, cord blood, and adult bone marrow (24). This could be due to differences in the sensitivity of the test or in the strategies used to purify the progenitor cells. In this regard we used FITC-labeled Abs directed against CD38 instead of PE-labeled CD38 reagents. As PE stains the cells brighter, it is possible that cells with a weak expression of CD38 were present in our starting population. It is still a matter of debate whether an uncommitted pluripotent precursor cell seeds the thymus and becomes T cell committed afterward or whether commitment occurs extrathymically and committed cells enter the thymus to complete their differentiation. Definitive proof awaits study of cells at the clonal level, but our findings suggest that CD34<sup>-</sup>CD38<sup>-</sup> fetal liver express RAG-1 and pTα mRNA reminiscent of committed T cells or that uncommitted cells could start the transcription of a variety of genes. Single-cell PCR will inform us on the frequency of these cells.

**Proposed model for development of different lineages in human thymus**

The kinetic study examining the acquisition of different lineage markers in our in vitro system, with particular emphasis on the earliest steps in differentiation, revealed the sequential expression of different surface Ags, shown in Fig. 9.

The starting population of fetal liver CD34<sup>-</sup>CD38<sup>-</sup> progenitor cells does not express the surface Ags CD1, CD2, CD3, CD4, CD5, or CD7. Analysis of this population revealed that the cells...
express CD45 but do not express CD45RA and a minority express CD45RA (<10%). The progenitor cells express predominantly c-kit (CD117) (>85%) and do not express CD10 and Thy-1 (CD90) (data not shown). CD3 is also not demonstrable in the cytoplasm. HLA-DR was already present on the starting population (>90%). Sorting for HLA-DR cells did not result in a different differentiation pattern, although the differentiating HLA-DR cells remain HLA-DR (our unpublished observations), with the exception of the dendritic cells that become HLA-DR+. The presence of pTα in the starting population is consistent with the concept of T-commitment of stem cells before the entry in the thymus or could indicate the presence of cells engaged in the extrathymic T cell pathway. At present, there is no direct evidence to conclude that one single progenitor cell in the thymus can differentiate into T, NK, and dendritic cells or whether there are different progenitor cells. Our observation that RAG-1 mRNA expression is absent after 3 days of FTOC is compatible with the view that the progenitor cells with RAG-1 mRNA transcripts are engaged in B cell differentiation and are not able to develop in FTOC.

It is clear that the first Ags that appear on the cell membrane of differentiating CD34+CD38−Lin− are CD38 and CD7 (Fig. 2). These markers are not lineage specific and are considered as activation markers. However, CD7 is not expressed by all cells, and we find evidence that strong expression of CD7 never occurs during differentiation of dendritic cells. This assumption is based on the fact that cyCD3 is very rapidly expressed within 3 days of culture on part of the CD3+CD7+ CD56+ cells, and that cyCD3 expression is almost exclusively found on cells with a strong CD7 expression. Cells with a lower CD7 expression might be precursor cells of cyCD3-positive cells. However, we cannot exclude that part of these cells express CD7 transiently and will never express cyCD3 and are cells at the earlier stages of the dendritic cell development. In this respect, we have been able to generate dendritic cells in FTOC starting from sorted CD34−CD7low− cells from fetal liver (data not shown). It is generally considered that expression of cyCD3 is an early marker for both NK and T cell commitment. As CD7 expression correlates with cyCD3 expression, CD7, in absence of membrane CD3 and CD4 expression, is an early T/NK marker in the thymus. The next Ag that is expressed is CD4. It is obvious, as shown in Fig. 3, that CD4 expression occurs in the absence of cyCD3 in these cells that will become dendritic cells. HLA-DR, which is already present from the start of the culture, is up-regulated during differentiation toward dendritic cells. We could never demonstrate CD5 on these cells, whereas a small fraction of these cells expresses CD1a (data not shown). Therefore, we can conclude that during differentiation toward dendritic cells, progenitor cells down-regulate CD34, possibly transiently express low levels of CD7, up-regulate HLA-DR and CD4, and never express cyCD3.

The next CD7+ population that can be detected expresses cyCD3, which is indicative for cells engaged in the T/NK cell pathway. At that time, CD4 is not expressed. When CD4 is expressed, HLA-DR is down-regulated (data not shown). Resort experiments have shown that these cells have lost the potential to generate dendritic cells, whereas they can generate both NK and T cells.

These data indicate that in humans the T/NK split occurs downstream of the NK/dendritic cell split. These data are compatible with the finding in human bipotential T/NK cells that have been demonstrated by Sanchez et al. (11). Others have shown that the human thymus contain CD34+ intrathymic precursors that develop into T and dendritic cells (35, 36). In our study, there is no evidence that dendritic cells are tightly linked to T cell development beyond a common precursor that is multipotent. This is in contrast to the observation in the mouse system, where a common T/dendritic cell progenitor was found (28). This could be due to species differences, difference between adult and fetal precursors, or to our model where an early precursor with myeloid potential is introduced. However, postnatal thymocytes sorted according to phenotypic differentiation markers and tested for their differentiation capacity in FTOC are consistent with the notion that dendritic cells branch off earlier that the T/NK bipotential cell progenitors. The CD34+CD1− subset, which is considered as the most immature thymic progenitor, gives rise to HLA-DR−CD4+CD3−, putative dendritic cells, T cells, and in the presence of IL-15 to cells with the CD56+ NK cell phenotype (data not shown). On the contrary, CD34+CD1+ thymic progenitors, considered to be at a later step of differentiation than the CD34+CD1− cells,
Finally, we find mature TCR-\(\alpha\beta\)ab maturation of the different cell populations. Our model was based on results show that our experimental model is able to support full maturation. This is could be due to a more elaborated selection process. These findings strongly support that the hybrid mouse-human FTOC is a netic and sequential order of phenotypes (data not shown). These studies results from starting CD34+CD38− populations from postnatal thymus gave the same kinematics and sequential order of phenotypes (data not shown). These findings strongly support that the hybrid mouse-human FTOC is a reliable model system for human thymocyte differentiation.

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References