Lymphoid and Myeloid Differentiation of Fetal Liver CD34$^+$Lineage$^-$ Cells in Human Thymic Organ Culture

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Summary

In this article, we report that the human fetal thymus contains CD34^{bright} cells (<0.01% of total thymocytes) with a phenotype that resembles that of multipotent hematopoietic progenitors in the fetal bone marrow. CD34^{bright} thymocytes were CD33$^{-/dull}$ and were negative for CD38, CD2, and CD5 as well as for the lineage markers CD3, CD4, and CD8 (T cells), CD19 and CD20 (B cells), CD56 (NK cells), glycophorin (erythrocytes), and CD14 (monocytes). In addition, total CD34$^+$ lineage negative (lin$^-$) thymocytes contained a low number of primitive myeloid progenitor cells, thus suggesting that the different hematopoietic lineages present in the thymus may be derived from primitive hematopoietic progenitor cells seeding the thymus. To investigate whether the thymus is permissive for the development of non-T cells, human fetal organ culture (FTOC) assays were performed by microinjecting sorted CD34$^+$lin$^-$ fetal liver cells into fragments of HLA-mismatched fetal thymus. Sequential phenotypic analysis of the FTOC-derived progeny of CD34$^+$lin$^-$ cells indicated that the differentiation into T cells was preceded by a wave of myeloid differentiation into CD14$^+$CD11b$^+$CD4^{dull} cells. Donor-derived B cells (CD19$^+$CD20$^+$) were also generated, which produced immunoglobulins (IgG and IgM) when cultured under appropriate conditions, as well as functional CD56$^+$CD3$^-$ NK cells, which efficiently killed K562 target cells in cytotoxicity assays. These results demonstrate that the microinjection of fetal liver hematopoietic progenitors into fetal thymic organ fragments results in multilineage differentiation in vitro.

A small percentage (<1%) of hematopoietic non-T cells are present in the human fetal and postnatal thymus. Thymic macrophages, monocytes, and dendritic cells can be readily identified in normal thymic sections (1–4) and they have been implicated in the complex selection process of immature thymocytes that give rise to a MHC-restricted peripheral T cell repertoire (5). In addition, NK and B cells have been detected in the human thymus (6–10), although there is no clear evidence demonstrating that thymic B cells and NK cells play a specific role in T cell differentiation.

The origin of the different hematopoietic lineages present in the thymus is presently unknown. During the late embryonic period in the human fetus, T cell progenitors generated in the liver migrate to the thymic rudiment at 7–9 wk of gestation (10). CD3$^+$ T cells can be detected in the fetal thymus (FT)[1] already at week 8 of gestation (11), although the FT is not anatomically completely differentiated until week 15 of gestation (12). It is possible that T cells and non-T cells present in the thymus are derived from a common multipotent hematopoietic progenitor (MHP). Alternatively, distinct progenitors committed to the lymphoid and myeloid lineages may migrate to the thymus, where they further differentiate into mature cells. It is, however, also possible that mature B, NK, and myeloid cells are initially generated in the hematopoietic organs and migrate from peripheral organs of the immune system to the thymus.

We reasoned that if the non-T cell thymic compartment develops intrathymically, we could expect to find a cellular population in the thymus with phenotypic and functional properties that resemble those of MHPs that have been described in the bone marrow (BM) and fetal liver (FL) (13, 14). Recent reports indicated that CD34$^+$CD38$^-$ fetal BM cells are enriched in early hematopoietic precursors. This population should also have T cell progenitor activity, since it includes Thy-1$^+$ cells (15) and CD34$^+$Thy-1$^+$ cells have been shown to contain T cell precursors (16). Taken together,

[1] *Abbreviations used in this paper:* BM, bone marrow; CFC, colony-forming cell; FL, fetal liver; FT, fetal thymus; FTOC, fetal thymic organ culture; HPP, high proliferative potential; LPP, low proliferative potential; MHP, multipotent hematopoietic progenitor; TN, triple negative; TRC, tricolor.

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these data indicate that $CD34^+CD38^-$ BM cells are enriched for MHP cells and may contain pluripotent stem cells. In the present study, we investigated the possible existence of a similar $CD34^+CD38^-$ lineage negative (lin^-) intrathymic population (lineage: $CD3^-CD4^-CD8^-CD14^-CD19^-CD20^-CD56^-$glycophorin$^-$) as well as the presence of early myeloid progenitors among $CD34^+lin^-$ fetal thymocytes.

The hypothesis that thymic non-T cells are derived from MHPs seeding the thymus would imply that this organ should be able to support the differentiation of non-T cell lineages. Appearance of myeloid and nonlymphoid cells together with thymocytes has been also reported in the SCID-hu system (17). However, it is currently unknown whether hematopoietic progenitor cells can develop into B and NK cells in the thymus. In the present study we investigated the ability of the human FT to support the differentiation of $CD34^+lin^-$ FL cells, a heterogeneous population containing both stem cells as well as lineage-committed progenitors, into T cells and non-T cell lineage cells (myeloid-, B-, and NK-cells) by using a human fetal thymic organ culture (FTOC) system.

Materials and Methods

mAbs. mAbs against the following markers were used: CD1 (CD1-RD, Coulter Corp., Hialeah, FL), CD2 (G11; Caltag Laboratories, San Francisco, CA), CD3 (anti-Leu-4), CD4 (anti-Leu-3a), CD5 (anti-Leu-1 and CD5-5D7, from Becton Dickinson & Co. [Mountain View, CA] and Caltag Laboratories, respectively), CD7 (anti-Leu-9), CD8 (anti-Leu-2a), CD11b (anti-Leu-15), CD13 (IOM13; Amac, Westbrook, ME), CD14 (anti-Leu-M9), CD16 (anti-Leu-11a), CD19 (anti-Leu-12), CD20 (anti-Leu-16), CD33 (anti-Leu-M9 and 251 from Becton Dickinson & Co. and Caltag Laboratories, respectively), CD34 (anti-HPCA.2), CD45 (anti-Hle-1) and anti-HLA-DR (anti-HLA-DR and HL38 from Becton Dickinson & Co. and Caltag Laboratories, respectively). All these mAbs were used conjugated to FITC, PE, tricolor (TRC) or biotinylated, as indicated in the legends to the figures and are from Becton Dickinson & Co., unless otherwise indicated. Anti-HLA-A2-FITC (CR11-351) and anti-HLA-A3-FITC (GAP-A3) were obtained from American Type Culture Collection (Rockville, MD). L307 (anti-B7) was kindly provided by Dr. L. Lanier (DNAX Research Institute). SPLV-3-FITC (anti-DQ) was generated in our laboratory. 89-FITC (anti-CD40) was kindly provided by Dr. G. Aversa (DNAX Research Institute). L185-FITC (anti-CD56) was kindly provided by Dr. J. Phillips (DNAX Research Institute). Streptavidin conjugated to allophycocyanin and streptavidin conjugated to TRC were purchased from Becton Dickinson & Co. and Caltag Laboratories, respectively.

Isolation of FL and FT Subpopulations. Human fetal tissues were 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 16 to 21 wk. FL and FT samples were homogenized through a wire mesh in the presence of RPMI containing 10% FCS. The isolation, by cell sorting using a FACStar® plus (Becton Dickinson & Co.), of >99% pure $CD34(PE)^+lin(FITC)^-$ FL cells and $CD34(FITC)^+lin(PE)^-$ triple negative (TN) fetal thymocytes was performed as described elsewhere (18).

High Proliferative Potential (HPP) Colony-Forming Cell (CFC) Assay. Myeloid progenitor cells were assayed as previously described (19). Cultures were scored for the presence of HPP-CFC (colonies >0.5 mm in diameter) and low proliferative potential (LPP)-CFC (colonies < than HPP-CFC but > than 50 cells/colony) (20).

Immunofluorescence and Flow Cytometry. Cell surface phenotypic analyses were performed as previously described (19).

FTOC. Human fetal thymic pieces containing 3–10 lobules were prepared, depleted of endogenous thymocytes and microinjected with 10^4 $CD34^+lin^-$ FL cells/piece sorted from an HLA-mismatched donor, as previously described (19). 20–40 microinjected pieces were placed back on the gelfoam rafts and cultured in Yssel's medium (21) supplemented with 1% human serum + 5% of FCS at 37°C in a humidified 5% CO_2 atmosphere. After 15 d in culture, 10 ng/ml of epidermal growth factor (Sigma Chemical Co., St. Louis, MO) was added to the FTOC medium to keep a good cellular viability of the thymic epithelial cells. At the indicated times, 5–10 injected thymic pieces, as well as the noninjected controls, were dispersed by gentle pipetting in PBS containing 5 mg/ml BSA and 0.2 mg/ml NaN_3 for further phenotypic analysis. In parallel to the FTOCs assays, $CD34^+lin^-$ FL cells were cultured at 10^4, 10^3, 10^2, and 10 cells/well to determine the putative contamination with mature T and NK cells, as described elsewhere (21, 22).

Culture of FTOC-derived Monocytes. Adherent cells from the bottom of the 6-well plates that contained the FTOC, were transferred to separated plates at day 15 after microinjection and cultured at 10^5 cells/well in Yssel's medium supplemented with 10% FCS and 40 ng/ml GM-CSF (kindly provided by Dr. R. Kastelein, DNAX Research Institute).

Culture Conditions for Induction of Ig Synthesis In Vitro. Cells derived from FTOC 15 d after injection with $CD34^+lin^-$ FL cells were cultured in duplicate at 1.5×10^4 cells/well in Yssel's medium supplemented with 10% FCS, in round bottomed plates (Linbro, McLean, VA) in the presence of 400 U/ml of IL-4 (Schering-Plough Research, Bloomfield, NJ). The $CD4^+$ T cell clone B21 was used 2–3 d after activation in the presence of feeder-cell mixture and PHA (Wellcome, Beckenham, Kent, UK), as previously described (21) and added to the FTOC-derived cells at 5,000 cells/well. After 14 d in culture, IgM, total IgG, IgA, and IgE secretion levels present in the culture supernatants were measured by ELISA as described elsewhere (23). The sensitivity levels of the IgM, total IgG, and IgA ELISAs were 0.5 ng/ml and the sensitivity of the IgE ELISA was 0.2 ng/ml.

In Vitro Expansion of FTOC-derived NK Cells. Day 15 FTOCs microinjected with $CD34^+lin^-$ FL cells were dispersed, and analyzed for the presence of donor-derived $CD56^+$ cells by cytofluorometry. The cellular suspension was cultured for 10 d in Yssel's medium supplemented with 1% human serum in the presence of 100 U/ml of rIL-2. For further expansion of the NK population, the cells were cultured for 15 d in the presence of feeder cells and PHA, as reported elsewhere (22).

Cytotoxicity Assays. The cytotoxicity assays were performed as described previously (21). The effector cells in these experiments were FTOC-derived NK cells or a peripheral blood-derived $CD56^+CD16^+CD3^-$ NK cell clone.

Results

Phenotypic Profile of $CD34^+lin^-$ Cells Present in the FT. $CD34^+$ TN thymocytes are characterized by the expression of T cell markers such as CD1, CD2, CD5, CD7, CD10, and CD28 (19, 24) and display T cell progenitor activity in a novel human FTOC system (18). To address the question of whether

a population phenotypically similar to primitive MHPs ($CD34^+CD38^-$ [13]) is present in the FT, $CD34^+$ TN thymocytes were depleted of lineage$^+$ cells and isolated by cell sorting from fetal thymi. Fig. 1 shows a three-color staining of sorted $CD34^+lin^-$ thymocytes. Electronic gates were set to analyze the expression of CD33, HLA-DR, CD2, and CD5 antigens on $CD34^{bright}CD38^-$ (R1, 1% of $CD34^+lin^-$ thymocytes), $CD34^{bright}$ $CD38^{dull}$ (R2, 6%), $CD34^{bright}$ $CD38^{bright}$ (R3, 14%), and $CD34^{dull}$ $CD38^{bright}$ (R4, 79%) thymocytes. As it has been reported for BM-derived hematopoietic stem cells (13), the small $CD34^+CD38^-lin^-$ thymic population (<0.01% of total thymocytes) expressed high levels of CD34 and did not express CD2 and CD5, suggesting that they are not yet committed to the T cell lineage. The expression of CD2 and CD5 was found to increase in parallel with the increase in CD38 expression and the decrease in CD34 expression. Interestingly, the $CD34^{bright}CD38^-$ thymocytes can be subdivided into two subsets, HLA-DR$^+$ and HLA-DR$^{-/dull}$. In addition, we consistently detected dull levels of CD33 expression among $CD34^{bright}$. These data suggest the existence of very early hematopoietic progenitors in the human FT.

Myeloid Potential of $CD34^+lin^-$ Fetal Thymocytes. Our recent studies on FL myeloid precursors have demonstrated that HPP-CFC, a compartment of primitive progenitors, are found predominantly among the $CD34^+CD38^-$ FL cells (14). Furthermore, as observed of the $CD34^+CD38^-$ thymic population, $CD34^+CD38^-$ FL counterpart cells comprised $CD33^+$ cells which could be subdivided into both HLA-DR$^+$ and HLA-DR$^-$ subsets. The $CD34^+CD38^-$ FL cells express higher levels of CD33 than their fetal thymic counterpart. A representative comparison (out of three experiments) of myeloid progenitor activity found among lin$^-$ FL and FT cells, obtained from a 16-wk-old fetus, revealed a far greater number of myeloid progenitors present in the FL as compared with the FT. The FL contained a total of 34,000 HPP-CFC and 77,000 (LPP-CFC) found at a frequency of 1,300 and $3{,}000/10^5$ lineage-depleted cells, respectively. The FT contained a total of only 91 HPP-CFC and 920 LPP-CFC which were present at the low frequency of 8.5 and $86/10^5$

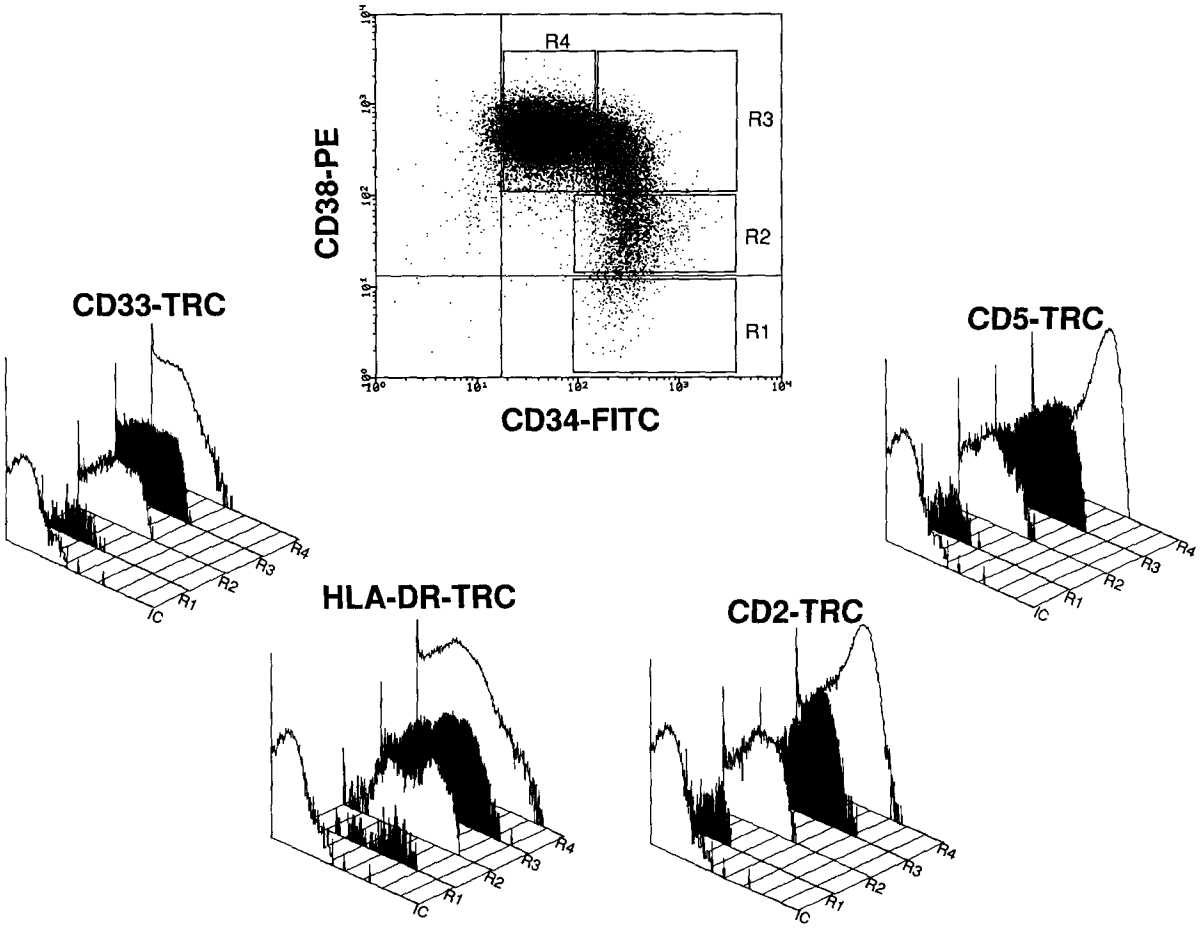

Figure 1. Three-color analysis of fetal $CD34^+lin^-$ TN thymocytes. Sorted $CD34^+lin^-$ TN thymocytes were stained with anti-CD38-PE and the indicated TRC-conjugated mAbs. 5×10^4 alive gated cells were acquired for a multicolor analysis on a FACScan®. Electronic gates were set to contain >95% of $CD34^{bright}CD38^-$ (R1), $CD34^{bright}CD38^{dull}$ (R2), $CD34^{bright}CD38^{bright}$ (R3), and $CD34^{dull}CD38^{bright}$ (R4). IC, isotype matched control. The respective mean intensities of fluorescence in regions R1, R2, R3, and R4 are: 11, 19, 13, and 4 for CD33; 221, 248, and 128, and 34 for HLA-DR; 6, 16, 50, and 70 for CD2; and 6, 13, 52, and 116 for CD5. The cell numbers (y-axis) in the histograms are presented on a logarithmic scale.

lineage-depleted cells, respectively. These results demonstrate that the FT contains both early and late myeloid progenitors, with the latter population containing the bulk of the thymic myeloid colony-forming potential and are consistent with the reported presence of CFU-C in immature populations of postnatal thymocytes (25).

The ability of the thymic microenvironment to sustain myelopoiesis was analyzed by comparing the in vitro growth of $CD34^+lin^-$ FL cells over 7 d in either cytokine-stimulated liquid cultures or after microinjection into fresh, undepleted thymic fragments. The number of HPP-CFC and LPP-CFC generated from $CD34^+lin^-$ FL cells during 7 d of growth in liquid culture stimulated by GM-CSF + KL + IL-3 + IL-6 increased 6- and 16-fold over starting values, respectively. In contrast, the numbers of HPP-CFC and LPP-CFC recovered from microinjected undepleted thymic fragments were, respectively, 20- and 5-fold less than in the freshly isolated FL cells. Thus, myeloid progenitor activity is only modestly maintained in FTOC relative to the potential of these progenitors in the presence of cytokines permissive for myelopoiesis.

FTOC Supports the Differentiation of $CD34^+lin^-$ FL Cells into Multilineage Progeny. To investigate the capacity of human FTOC to support the differentiation of non-T cell lineages, we used a source of MHP cells that do not contain committed T cell precursors comparable to those found in the FT (19). The $CD34^+lin^-$ subset represents 3.8 ± 1.6% (n = 8) of mononuclear glycophorin$^-$ FL cells, the phenotype of which has been described previously (18). Although not shown, sorted $CD34^+lin^-$ FL cells (>99% pure) did not express mRNA for RAG-1 and RAG-2 genes, as determined by PCR (Bárcena, A., and H. Spits, unpublished observations), indicating the lack of TCR and Ig gene rearrangement (26, 27). Furthermore, $CD34^+lin^-$ FL cells did not give rise to T or NK cells when grown in the presence of irradiated feeders + PHA + IL-2. These data support the notion that the $CD34^+lin^-$ FL cells lack progenitors committed to NK, B, and T cell lineages.

10^4 $CD34^+lin^-$ FL cells sorted (>99% pure) from an HLA-$A2^+$ donor were microinjected into 20–40 individual fetal thymic pieces (HLA-$A2^-$). Phenotypic analysis of the cellular progeny was performed at different periods of culture to search for the presence of surface markers characteristic of different hematopoietic lineages. The cellular recovery was typically in the range of 2–5 × 10^4 cells/thymic piece with a 82–95% cellular viability. As it is shown in Table 1, two populations bearing the donor HLA and showing overlapping but distinct forward vs. side scatter FACS® profile were detected and electronically gated in all phenotypic analysis. At day 15 in FTOC, most of the cells recovered from the thymic pieces were donor-derived (HLA-$A2^+$), that showed a heterogeneous lymphoblastic and monoblastic morphology. The large cells were mostly $CD33^+$ and $CD4^+$ and some expressed markers found on NK cells (CD56), B cells (CD19 and CD20), and thymocytes (CD1, CD4, and CD8) (Table 1). The analysis of the lymphoid population revealed a lower expression of CD33 and a higher expression of the thymic markers CD1, CD3, and CD8. At later time points of FTOC (days 20 and 25), the cellular distribution was reversed. The proportion of large $CD33^+$ cells was decreased, correlating with an increase of cells of the lymphoid type as well as in the expression of T and B cells markers (Table 1). At day 25, cells bearing lymphoid markers were also present in the large cell population, which is due likely to the existence of a small proportion of lymphoblasts that were consistently observed during cell counting at late

Table 1. *Kinetics of Reconstitution in FTOC Injected with $CD34^+$ lin^- Fetal Liver Cells*

Day	Number of cells/piece	Percent HLA-$A2^+$ cells	Percentage of cells expressing						
			CD3	CD4	CD8	CD1	CD56	CD19	CD33
			Large cells						
15	35,000	94	1.5	63	2	17	14	32	79
20	5,000	99	2	40	2	ND*	27	20	ND
25	4,000	99	37	65	31	80	18	10	17
			Small cells						
15	17,000	85	5.7	65	10	57	15	3	31
20	20,000	88	10	67	36	ND	8	5	ND
25	37,000	95	60	75	73	92	15	10	5

Fetal thymic fragments (HLA-$A2^-$) were microinjected with 10^4 sorted $CD34^+lin^-$ cells from an HLA-$A2^+$ FL. After the indicated days in culture, the thymic fragments were homogenized, and cell counts were obtained. The recovered cells were stained and subjected to FACS® analysis, as described in the Materials and Methods. The results, representative of four independent experiments, are expressed as the percentage of positive cells over isotype-matched controls. Gate was set to contain 95 and 98% of large and small cells, respectively. Large cells showed a high profile in FSC vs. SSC plot, corresponding to large, granular cells. Small cells were typically found in the lymphoid gate. *ND, not done.

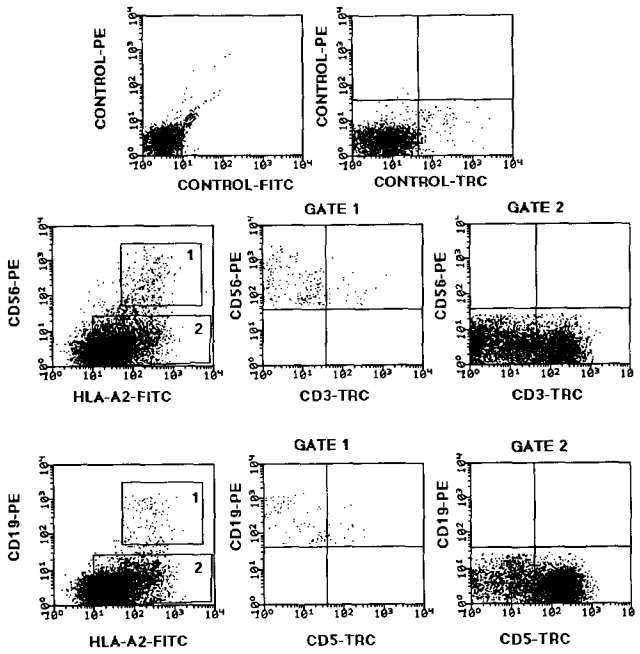

Figure 2. Three-color analysis of day 25 FTOC-derived progeny. Sorted $CD34^+lin^-$ cells from an $HLA\text{-}A2^+$ FL were microinjected into $HLA\text{-}A2^-$ FT fragments, as described in Materials and Methods. After 25 d in culture, the FT fragments were dispersed. The harvested cells were incubated with the indicated mAbs and 5×10^3 cells were acquired for three-color analysis using a FACScan®.

timepoints in FTOC. The early appearance of $CD33^+$ immature myeloid cells before the emergence of lymphoid population suggests a biphasic differentiation of $CD34^+lin^-$ cells. Although not included in Table 1, at day 17 in FTOC we observed that 65% of the large cells were $CD14^+$ and 83% expressed CD33. The percentage of $CD56^+$ (5–15% of the $CD34^+lin^-$ progeny) was very similar in both large and small cell subpopulations and it varied very little during the course of FTOC. Since activated T cells can express CD56, three-color analyses of day 25 harvested FTOC cells were performed. Fig. 2 shows that donor-derived $HLA\text{-}A2^+$ $CD56^+$ cells did not express CD3, whereas the majority of $HLA\text{-}A2^+$ $CD56^-$ cells were $CD3^+$. In addition, Fig. 2 shows that the $CD19^+$ cells generated in FTOC are $HLA\text{-}A2^+$, expressing low to negative levels of CD5. Taken together, the results shown in Table 1 and Fig. 2 clearly demonstrate the generation of myeloid, T, NK, and B cells in FTOC.

Generation of Monocytes in FTOC. As early as day 7 after the microinjection of $CD34^+lin^-$ FL cells into FTOC, monocytic-, and myeloblastoid cells were observed at the bottom of the plates in which the FTOC were cultured. Cells with a dendritic or interdigitating cell morphology were also present at a low frequency. Fig. 3 shows the characteristic elongated and adherent morphology of monocytes, as well as the round small myelomonocytic immature cells. For further expansion of the FTOC-derived monocytes, the cells were transferred to separate plates and cultured up to 4 wk in the presence of GM-CSF. Fig. 4 shows the phenotypic profile of FTOC-derived monocytes after 20 d in culture. All cells were $HLA\text{-}A2^+$, indicating the FL origin, and most of them were $CD11b^+$. The level of HLA-DR expression was quite variable among different experiments, increasing at later time points in culture (data not shown). Fig. 4 shows dull HLA-DR expression on 25% of the cells. The majority of these cells expressed $CD4^{dull}$ and about 50% of the cells were $CD14^+$. Although 25% of the cells were $CD33^+$, the expression of the CD34 marker was negative, suggesting that myeloid precursors ($CD33^+CD34^+$) present in the original $CD34^+lin^-$ population were undergoing in vitro differentiation into $CD33^+CD34^-$ cells and, moreover, some of them completed the differentiation pathway into $CD14^+$ cells. T cells ($CD3^+$), B cells ($CD19^+$), or NK cells ($CD56^+$) were not present in the medium of FTOCs (Fig. 4). Since we occasionally observed the appearance of a small proportion of cells with a cellular morphology similar to dendritic cells (Fig. 3), we also investigated the expression of some dendritic cell markers on the FTOC-derived cells. Fig. 4 shows that most of the cells were positive for HLA-DQ and expressed CD1a antigen in a dull fashion, but we observed a small proportion of cells expressing CD1a and high levels of HLA-DQ, as it has been described for dendritic cells (28). Few $CD14^+$ cells were found to be $B7^+$, but a significant proportion (20%) coexpressed CD40. These data suggest the

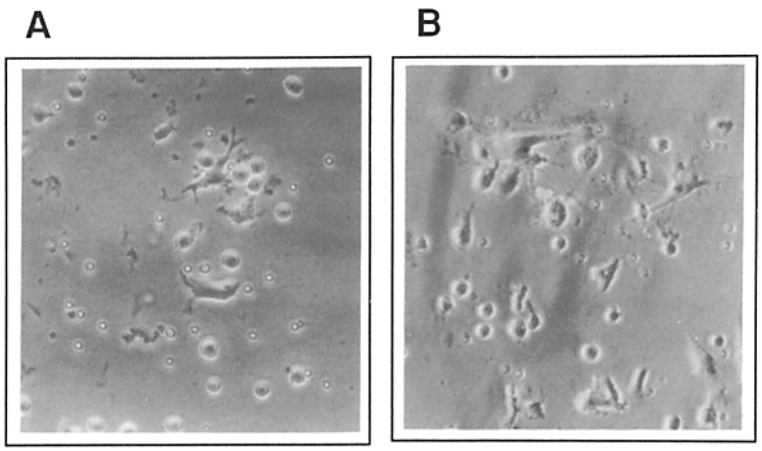

Figure 3. Morphology of FTOC-derived myelomonocytic cells. Microphotographs of thee myelomonocytic cells (original magnification ×400) observed at day 7 (*A*) and day 17 (*B*) at the bottom of the plates in which the thymic fragments were incubated.

generation of not only monocytes, but a significant proportion (20%) coexpressed CD40. These data suggest the generation of not only monocytes, but also dendritic cells in FTOC. No granulocytes ($CD13^+$) were observed in our cultures. The monocytes generated in FTOC were capable to induce the proliferation of PBL in MLR assays at comparable levels to those induced by sorted $CD14^+$ monocytes from peripheral blood, thus demonstrating their functional capabilities (data not shown).

Generation of Functional B Cells in FTOC. We have previously shown that FL B cells undergo Ig isotype switching and differentiation into Ig-secreting cells when cultured in the presence of IL-4 and the activated $CD4^+$ T cell clone B21 (29). To investigate the functional maturity of the $CD19^+$ cells generated in the FTOC (Table 1 and Fig. 2), cellular suspensions obtained from FTOC at day 15 were cultured under these conditions. Freshly isolated $CD34^+lin^-$ FL cells failed to differentiate into Ig-secreting cells under these culture conditions (Table 2). However, when day 15 progeny of $CD34^+lin^-$ FL cells were recovered from FTOC and then cultured in the presence of B21 cells + IL-4, low but detectable levels of IgM synthesis were observed (Table 2). Since IgG production was also observed, these naive and unprimed FTOC-derived B cells are capable of undergoing Ig isotype switching, demonstrating the functional maturity of these cells.

Generation of Functional NK Cells in FTOC. As shown in Table 1 and Fig. 2, $CD56^+$ cells were detected as early as 15 d after injection of $CD34^+lin^-$ FL cells. Due to the low number of $CD56^+$ cells present in the FTOC (5–15%), a direct functional analysis of this population was not possible. Therefore, a subsequent in vitro expansion of these cells, as described in Materials and Methods, was required to assess the functional status of these cells. At day 15, very few mature T cells had been generated in FTOC (Table 1), allowing the establishment of cultures of NK cells in the presence of rIL-2 without contaminating T or myeloid cells. After in vitro expansion of the FTOC-derived cells, most of the cells displayed the donor HLA haplotype, >80% of the cells expressed CD56 and ~30% coexpressed CD16 (Fig. 5). In 50% of the experiments a small and variable percentage of donor-derived mature $CD3^+$ cells was observed. The $CD56^+$ cells generated in FTOC displayed NK activity against K562 at all the effector/target ratios tested (Fig. 6). The positive control for these experiments was a $CD56^+$ NK clone derived from peripheral blood. Cytotoxicity assays performed with the freshly isolated $CD34^+lin^-$ FL cells indicated the absence of NK activity (data not shown), demonstrating the in vitro differentiation of these cells into functionally and phenotypically mature NK cells.

Discussion

In the present study we investigated whether the human FT contains primitive hematopoietic progenitors. We show that sorted $CD34^+lin^-$ FT cells contains a very small population of $CD34^{bright}CD38^-$ cells (0.5–1% of $CD34^+lin^-$ thymocytes). The phenotypic profile of these cells resembles

Table 2. *Induction of Ig Synthesis by Cells Derived from FTOC*

	Ig isotype produced		
	IgM	IgG	IgE
Exp. 1		*ng/ml*	
Medium	<0.5	<0.5	<0.2
B21 + IL-4	<0.5	14.0 ± 3.8	<0.2
Exp. 2			
Medium	<0.5	<0.5	<0.2
B21 + IL-4	3.7 ± 0.7	3.5 ± 5.4	<0.2
B21 + IL-4 (noninjected FTOC)	<0.5	<0.5	<0.2
B21 + IL-4 ($CD34^+lin^-$ cells, day 0)	<0.5	<0.5	<0.2
Exp. 3			
Medium	<0.5	<0.5	<0.2
B21 + IL-4	3.2 ± 0.1	4.7 ± 1.3	<0.2
B21 + IL-4 (noninjected FTOC)	<0.5	<0.5	<0.2
B21 + IL-4 ($CD34^+lin^-$ cells, day 0)	<0.5	<0.5	<0.2

Cells derived from FTOC 15 d after injection with $CD34^+lin^-$ cells were cultured at 15×10^3 cells/well in duplicate in the presence or absence of IL-4 (400 U/ml) and B21 cells (5×10^3 cells/well). As a control, cells derived from FTOC not injected with $CD34^+lin^-$ cells and freshly isolated $CD34^+lin^-$ cells were cultured in parallel in experiments 2 and 3. The values represent the mean ± SD of Ig concentrations (ng/ml) detected by ELISA after a culture period of 14 d.

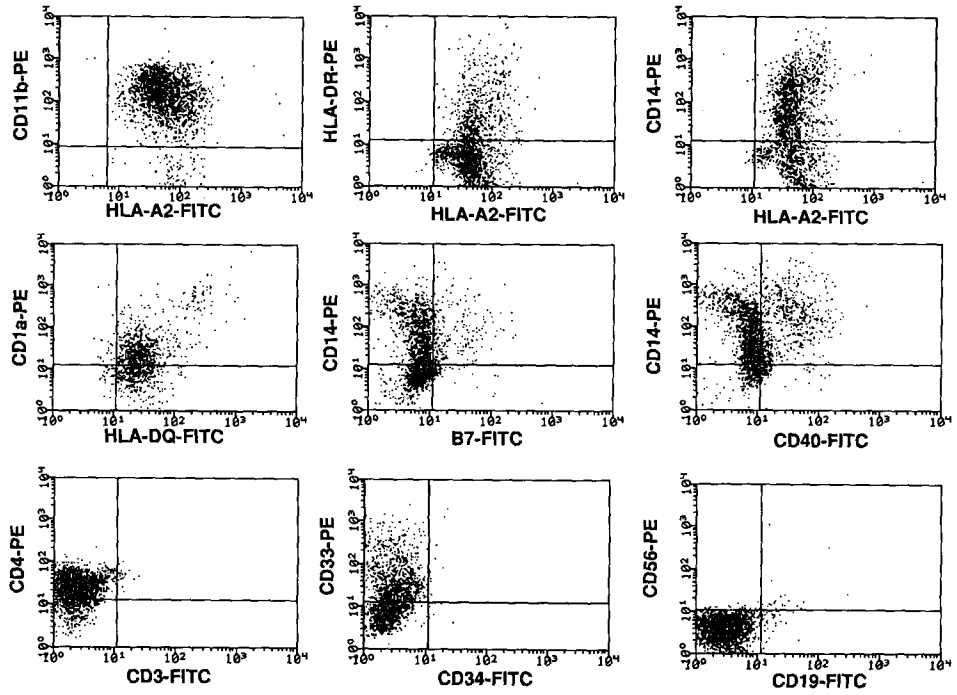

Figure 4. Phenotypic analysis of FTOC-derived myelomonocytic cells. HLA-A2$^+$ CD34$^+$lin$^-$ FL sorted cells were microinjected into HLA-A2$^-$ thymic fragments. As described in Materials and Methods, after 15 d in FTOC and 5 d in culture in the presence of 40 ng/ml of GM-CSF, the cells were stained with the indicated mAbs and subjected to two-color analysis on a FACScan®. The quadrants were set to contain >98% of the isotype matched FITC and PE control mAbs.

that of CD34$^+$CD38$^-$ FL cells (14) and fetal BM cells (13, 30) described to be highly enriched for hematopoietic stem cells, since they contain HLA-DR$^+$ and HLA-DR$^{-/dull}$ cells and express CD33$^{-/dull}$. The thymic CD34^{bright}CD38$^-$ cells are probably not yet committed to the lymphoid lineages, since they do not express the T/NK cells marker CD2 or the T/B cell marker CD5. Our results are in contrast with those of Terstappen et al. (31), that did not detect these intrathymic cells. An explanation for this discrepancy is that these authors analyzed total unseparated thymocytes by means of multiparametric fluorescence studies. The CD34^{bright}CD38$^-$ thymocytes comprise <0.01% of the total thymocytes, well below the reported sensitivity threshold of $1/10^4$ cells.

The notion that the human FT contains a low number of hematopoietic progenitor cells is consistent with observations obtained in SCID-hu model (17). SCID-hu mice co-transplanted with FL and FT showed long-term T cell reconstitution, whereas >90% of the mice transplanted with FT alone did not exhibit T cell reconstitution. However, it was also reported that <10% of the SCID mice transplanted with FT alone exhibited long-term thymopoiesis and these thymi contained the BM-like areas, similar to when FL and FT are transplanted together. Our observation of HPP-CFC in the FT reinforces the notion that this organ contains primitive, possibly MHPs. We have previously shown that HPP-CFC, an early progenitor compartment, is enriched in the CD34^{bright}CD38$^-$CD33$^+$lin$^-$ population of FL cells (14). In this study, we observed a low, but detectable number of HPP-CFC and LPP-CFC in the FT, which is consistent with the rarity of CD34^{bright}CD38$^-$lin$^-$ thymocytes that are CD33$^{-/dull}$. The phenotypic profile of these cells as well as

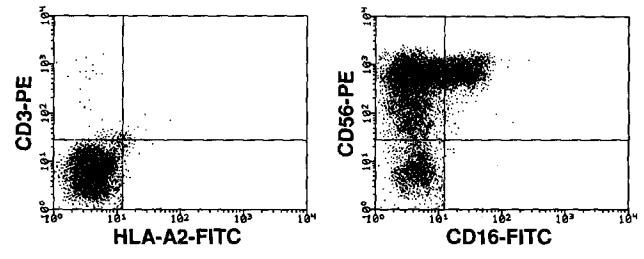

Figure 5. Phenotypic analysis of FTOC-derived NK cells. HLA-A2$^-$ CD34$^+$lin$^-$ FL sorted cells were microinjected into HLA-A2$^+$ thymic fragments. After 15 d in culture, the thymic fragments were dispersed and 2–5 × 10^4 cells were cultured as described in Materials and Methods and then subjected to two-color analysis using a FACScan®. The quadrants were set to include 95% of the isotype matched FITC and PE control mAbs.

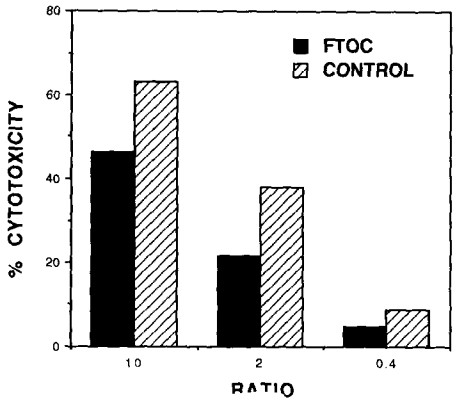

Figure 6. Cytotoxic activity of FTOC-derived NK cells. Day 15 FTOCs injected with CD34$^+$lin$^-$ FL cells were dispersed and expanded in vitro as described in Materials and Methods. The cells were then assayed in triplicate wells for cytotoxic activity against K562 cell line at the indicated effector/target ratios. The control is a NK clone derived from peripheral blood (CD56$^+$CD16$^+$CD3$^-$).

the low number of HPP-CFC present in the FT strongly suggest that the $CD34^{bright}CD38^{-}lin^{-}$ thymic population might contain progenitors for all the hematopoietic lineages present in the human thymus. To investigate this possibility requires the isolation and direct functional analysis of $CD34^{bright}CD38^{-}lin^{-}$ thymic population, which was, however, prevented by the paucity of these cells. Therefore, we cannot yet conclude that this thymic subset displays pluripotent hematopoietic activity.

The existence of an intrathymic cell population with a phenotype of MHPs has also been found in the mouse. A $Thy\text{-}1^{low}CD4^{low}$ population ($CD4^{low}$ precursor) in the adult mouse thymus (32), with a phenotype that resembles that of murine hematopoietic stem cells as identified by several groups in the murine BM (33, 34). The thymic $CD4^{low}$ precursors differentiated into T and dendritic cells following intrathymic injection and into B cells after intravenous injection in congenic recipients (35, 36), but, in contrast with $Thy\text{-}1^{low}CD4^{low}$ BM cells, they are not totipotent because they did not generate all the hematopoietic lineages. A recent report shows the presence of a $c\text{-}kit^{+}Thy\text{-}1^{low}lin^{-}$ cells in the adult murine thymus (37), that generates T, B, and $NK1.1^{+}$ cells after intravenous injection, but no myeloid and erythroid cells were detected, suggesting that this population is committed to the lymphoid lineages. Since these studies only analyzed the adult thymus, they do not preclude the presence of MHP in the FT. It might be possible that early in ontogeny, the thymus is seeded with MHP. During later fetal stages and after birth, these cells may recede and bipotent (giving rise to T and dendritic cells), but no pluripotent, hematopoietic precursors would function as progenitors for T and non-T thymic cells.

The presence of primitive hematopoietic progenitor in the thymus raises the possibility that the different hematopoietic lineages found in the thymus can be derived from a common precursor. In that case, the thymic microenvironment should be permissive to the development of multilineage progeny from hematopoietic progenitors. Indeed, here it is demonstrated that, in the absence of exogenous cytokines, the FTOC system provides the signals necessary to support the differentiation of purified immature $CD34^{+}lin^{-}$ FL cells into all members of the lymphoid lineages, T, B, and NK cells and into myelomonocytic cells.

The kinetics of development of the different lineages in the FTOC were different. The number of myelomonocytic cells peaks at early time points in culture and is followed by the migration of these cells out of the thymic fragments and an increase of the number of lymphoid cells within the thymic fragments. It is important to stress the fact that no exogenous growth factors were added to the FTOC. Thus, the in vitro maturation of myeloid cells was presumably sustained by myeloid differentiation factors produced in FTOC. This is very reminiscent of the intrathymic reconstitution of lethally irradiated mice with purified mouse stem cells, where it was observed that, in the first stages, thymi were repopulated with the donor-derived myeloid cells, then as their numbers went down, the donor T cells started increasing (38). An interesting question derived from these observations is whether the development of MHP cells into T cells in the thymus requires a prior generation of myeloid cells. It can be hypothesized that replenishment of thymic accessory cells, such as dendritic cells and monocytes, might have a positive influence on the development of T cell progenitors. In addition, the finding that FTOC supports myelopoiesis is consistent with the reported production by thymic epithelial cells of multiple cytokines associated with the growth of myeloid cells (39–41) as well as the capacity of these cells to support myeloid cell growth (42).

That the thymus can support the development of NK and B cells has not before been appreciated. It has been previously shown that the thymus contains not only mature and functional NK cells (6), but also NK precursors ($CD56^{-}$ $CD16^{-}$) (43), suggesting that NK development might take place intrathymically. This idea is strongly supported by our results, showing that human FTOC induces in vitro differentiation of FL MHPs into mature NK cells. Moreover, freshly isolated FL MHPs did not display cytotoxic activity, demonstrating that their differentiation into FTOC is required for the acquisition of functional capabilities. Human thymic B cells have been shown to be phenotypically and functionally distinct from B cells derived from peripheral immune organs, since 50% of thymic B cells express CD2 and CD5 and they are unresponsive to anti-CD40 mAbs (8, 9). Here, we demonstrate that the FTOC supports the in vitro differentiation of $CD34^{+}lin^{-}$ FL cells into functionally mature B cells, which can be induced in vitro to produce IgM and IgG. Altogether, our data support that the thymic B cell compartment is not derived from circulating B cells, and it might be generated from MHPs seeding the thymus.

An important question arising from our studies is why the thymic microenvironment is devoted to preferentially support T cell development, despite its intrinsic capability to support the development of other hematopoietic lineages. It can be proposed that the developing T cells in the FT may influence their own differentiation by controlling the development of hematopoietic precursors into other lineages. In fact, we show that the microinjection of FL $CD34^{+}lin^{-}$ cells into fresh FT fragments that were undepleted of endogenous thymocytes, resulted in a rapid decrease of myeloid progenitors, while the short-term culture of the same cells in the presence of cytokines increased the number of progenitors. Therefore, the presence of T cells in the thymus can inhibit extensive in vitro myelopoiesis. This inhibitory effect could in part be due to T cell derived factors such as IL-4 and IFN-γ, which are constitutively expressed in the human thymus (44, 45) and strongly inhibited the production of myeloid differentiation factors G-CSF and GM-CSF by IL-1–stimulated thymic epithelial cells (40, 46). In addition, IFN-γ induces HLA-DR expression and upregulates intercellular adhesion molecule 1 (ICAM-1) expression on thymic epithelial cells (40). These events might positively influence T cell development. Thus, developing thymic T cells and the thymic stroma might cooperate to regulate cytokine production that control further development of myeloid cells in the physiologic thymic environment. Our FTOC system provides the opportunity to test this hypothesis.

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References

1. Janossy, G., M. Bofill, L.W. Poulter, E. Rawlings, G.D. Burford, C. Navarrette, A. Ziegler, and K. Kelemen. 1986. Separate ontogeny of two macrophage-like accessory cell populations in the human fetus. *J. Immunol.* 136:4354.
2. Landry, D., M. Lafontaine, M. Cossette, C. Barthélémy, C. Chartrand, S. Montplaisir, and M. Pelletier. 1988. Human thymic dendritic cells. Characterization, isolation and functional assays. *Immunology.* 65:135.
3. Ruco, L.P., S. Rosati, F. Monardo, E. Pescarmona, E.A. Rendina, and C.D. Baroni. 1989. Macrophages and interdigitating reticulum cells in normal thymus and in thymoma: an immunohistological study. *Histopathology (Oxf.).* 14:37.
4. Kampinga, J., P. Nieuwenhuis, B. Roser, and R. Aspinall. 1990. Differences in turnover between thymic medullary dendritic cells and a subset of cortical macrophages. *J. Immunol.* 145:1659.
5. Lo, D., and J. Sprent. 1986. Identity of cells that imprint H-2 restricted T-cell specificity in the thymus. *Nature (Lond.).* 319: 672.
6. Mingari, M.C., A. Poggi, R. Biassoni, R. Bellomo, E. Ciccone, N. Pella, L. Morelli, S. Verdiani, A. Moretta, and L. Moretta. 1991. In vitro proliferation and cloning of $CD3^-$ $CD16^+$ cells from human thymocyte precursors. *J. Exp. Med.* 174:21.
7. Lanier, L.L., H. Spits, and J.H. Phillips. 1992. The developmental relationships between NK and T cells. *Immunol. Today.* 13:392.
8. Punnonen, J., and J.E. de Vries. 1993. Characterization of a novel $CD2^+$ human thymic B-cell subsets. *J. Immunol.* 151: 100.
9. Isaacson, P.G., A.J. Norton, and B.J. Addis. 1987. The human thymus contains a novel population of B lymphocytes. *Lancet.* ii:1488.
10. Lobach, D.F., and B.F. Haynes. 1987. Ontogeny of the human thymus during fetal development. *J. Clin. Immunol.* 7:81.
11. Furley, A.J., S. Mizutani, K. Weilbaecher, H.S. Dhaliwal, A.M. Ford, L.C. Chan, H.V. Molgaard, B. Toyonaga, T. Mak, P. van den Elsen, et al. 1986. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. *Cell.* 46:75.
12. Haynes, B.F. 1984. Phenotypic characterization and ontogeny of the human thymic microenvironment. *Clin. Res.* 32:500.
13. Terstappen, L.W.M.M., S. Huang, M. Safford, P.M. Landsdorp, and M.R. Loken. 1991. Sequential generation of hematopoietic colonies derived from single nonlineage-committed $CD34^+CD38^-$ progenitor cells. *Blood.* 77:1218.
14. Muench, M.O., J. Cupp, J. Polakoff, and M.G. Roncarolo. 1994. Characterization of CD33, CD38 and HLA-DR expression on human fetal liver progenitors with a high proliferative potential. *Blood.* In press.
15. Craig, W., R. Kay, R.L. Cutler, and P.M. Lansdorp. 1993. Expression of Thy-1 on human hematopoietic progenitor cells. *J. Exp. Med.* 177:1331.
16. Baum, C.M., I.L. Weissman, A.S. Tsukamoto, A.M. Buckle, and B. Peault. 1992. Isolation of a candidate human hematopoietic stem-cell population. *Proc. Natl. Acad. Sci. USA.* 89:2804.
17. Namikawa, R., K.N. Weilbaecher, H. Kaneshima, E.J. Yee, and J.M. McCune. 1990. Long-term hematopoiesis in the SCID-hu mouse. *J. Exp. Med.* 172:1055.
18. Galy, A., S. Verma, A. Bárcena, and H. Spits. 1993. Precursors of $CD3^+CD4^+CD8^+$ cells in the human thymus are defined by CD34. Delineation of early events in human thymic development. *J. Exp. Med.* 178:391.
19. Bárcena, A., M.O. Muench, A.H.M. Galy, J. Cupp, M.G. Roncarolo, J.H. Phillips, and H. Spits. 1993. 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. *Blood.* 82:3401.
20. Bradley, T.R., and G.S. Hodgson. 1979. Detection of primitive macrophage progenitor cells in mouse bone marrow. *Blood.* 54:1446.
21. Yssel, H., J.E. De Vries, M. Koken, W. van Blitterswijk, and H. Spits. 1984. Serum-free medium for the generation and the propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods.* 72:219.
22. Phillips, J.H. T. Hori, A. Nagler, N. Bhat, H. Spits, and L.L. Lanier. 1992. Ontogeny of human natural killer (NK) cells: fetal NK cells mediate cytolytic function and express cytoplasmic CD3 ϵ, δ proteins. *J. Exp. Med.* 175:1055.
23. Péne, J., F. Rousset, F. Briére, I. Chétien, J.-Y. Bonnefoy, H. Spits, T. Yokota, K.-I. Arai, J. Banchereau, and J.E. de Vries. 1988. IgE regulation by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E2. *Proc. Natl. Acad. Sci. USA.* 85:6880.
24. Toribio, M.L., J.M. Alonso, A. Bárcena, J.C. Gutiérrez, A. de la Hera, M.A.R. Marcos, C. Márquez, and C. Martínez-A. 1988. Human T-cell precursors: involvement of the IL-2 pathway in the generation of mature T cells. *Immunol. Rev.* 104:55.
25. Kurtzberg, J., S.M. Denning, L.M. Nycum, K.H. Singer, and

B.F. Haynes. 1989. Immature human thymocytes can be driven to differentiate into nonlymphoid lineages by cytokines from thymic epithelial cells. *Proc. Natl. Acad. Sci. USA.* 86:7575.
26. Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannous. 1992. RAG-1 deficient mice have no mature B and T lymphocytes. *Cell.* 68:869.
27. Shinkai, Y., G. Rathbun, K.-P. Lam, E.M. Oltz, V. Stewart, M. Datta, F. Young, A.M. Stall, and F.W. Alt. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell.* 68:855.
28. Freudenthal, P.S., and R.M. Steinman. 1990. The distinct surface of human blood dendritic cells, as described after an improved isolation method. *Proc. Natl. Acad. Sci. USA.* 87:7698.
29. Punnonen, J., G.G. Aversa, B. Vandekerckhove, M.-G. Roncarolo, and J.E. de Vries. 1992. Induction of isotype switching and Ig production by $CD5^+$ and $CD10^-$ human fetal liver B cells. *J. Immunol.* 148:3398.
30. Huang, S., and L.W.M.M. Terstappen. 1992. Formation of haematopoietic microenvironment and haemoatopoietic stem cells from single bone marrow cells. *Nature (Lond.).* 360:745.
31. Terstappen, L.W., S. Huang, and L.J. Picker. 1992. Flow cytometric assessment of human T-cell differentiation in thymus and bone marrow. *Blood.* 79:666.
32. Wu, L., M. Antica, G.R. Johnson, R. Scollay, and K. Shortman. 1991. Developmental potential of the earliest precursor cells from the adult mouse thymus. *J. Exp. Med.* 174:1617.
33. Frederickson, G.G., and R.S. Basch. 1989. L3T4 antigen expression by hematopoietic cells. *J. Exp. Med.* 169:1473.
34. Spangrude, G., C.E. Mueller-Sieburg, S. Heimfeld, and I.L. Wiessman. 1988. Two rare populations of mouse Thy-1^{lo} bone marrow cells repopulate the thymus. *J. Exp. Med.* 167:1671.
35. Ardavin, C., L. Wu, C.-L. Li, and K. Shortman. 1993. Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature (Lond.).* 362:761.
36. Wu, L., R. Scollay, M. Egerton, M. Pearse, G.J. Spangrude, and K. Shortman. 1991. CD4 expressed on earliest T-lineage precursor cells in the adult murine thymus. *Nature (Lond.).* 349:71.
37. Matsuzaki, Y., J.-i. Gyotoku, M. Ogawa, S.-i. Nishikawa, Y. Katsura, G. Galechin, and H. Nakauchi. 1993. Characterization of c-*kit* positive intrathymic stem cells that are restricted to lymphoid differentiation. *J. Exp. Med.* 178:1283.
38. Spangrude, G.J., and R. Scollay. 1990. Differentiation of hematopoietic cells in irradiated mouse thymic lobes: kinetics and phenotype of the progeny. *J. Immunol.* 145:3661.
39. Galy, A.H.M., H. Spits, and J.A. Hamilton. 1993. Regulation of M-CSF production by cultured thymic epithelial cells. *Lymphokine and Cytokine Research* 12:265.
40. Galy, A.H.M., and H. Spits. 1991. IL-1, IL-4, and IFN-γ differentially regulate cytokine production and cell surface molecule expression in culture thymic epithelial cells. *J. Immunol.* 147:3823.
41. Le, P.T., S. Lazorick, L.P. Whichard, Y.C. Yang, S.C. Clark, B.F. Haynes, and K.H. Singer. 1990. Human thymic epithelial cells produce IL-6, granulocyte-monocyte-CSF and leukemia inhibitory factor. *J. Immunol.* 145:3310.
42. Izon, D.R., R.L. Boyd, G.A. Waanders, and A. Kelso. 1989. The myelopoietic inducing potential of mouse thymic stroma cells. *Cell. Immunol.* 124:264.
43. Sánchez, M.J., H. Spits, L.L. Lanier, and J.H. Phillips. 1993. Human natural killer cell committed thymocytes and their relation with the T cell lineage. *J. Exp. Med.* 178:1857.
44. Bárcena, A., M.J. Sánchez, J.L.d.l. Pompa, M.L. Toribio, G. Kroemer, and C. Martínez-A. 1991. Involvement of the interleukin 4 pathway in the generation of functional gamma/delta cells from human pro-T cells. *Proc. Natl. Acad. Sci. USA.* 88: 7689.
45. Vandekerckhove, B.A.E., A. Bárcena, D. Schols, S. Mohan-Peterson, H. Spits, and M.-G. Roncarolo. 1994. In vivo cytokine expression in the thymus. CD3high human thymocytes are activated and already functionally differentiated in helper and cytotoxic cells. *J. Immunol.* 152:1738.
46. Pelus, L.M., O.G. Ottman, and K.H. Nocka. 1988. Synergistic inhibition of human marrow granulocyte-macrophage progenitor cells by prostaglandin E and recombinant interferon-α, β and γ and an effect mediated by tumor necrosis factor. *J. Immunol.* 140:479.