Precursors of $CD3^+CD4^+CD8^+$ Cells in the Human Thymus Are Defined by Expression of CD34. Delineation of Early Events in Human Thymic Development

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Summary

Studies of the most immature T cell progenitors in the human thymus have been hampered by the lack of markers and assays that define these cells. In this report we used a novel human fetal thymic organ culture system to determine the potential of T cell precursors isolated from human postnatal thymus, to differentiate into $CD3^+$ thymocytes, and to investigate early stages of human T cell development. It was found that thymocytes that lack the markers CD3, CD4, and CD8 (triple negative [TN]) can differentiate in an allogeneic organotypic thymic culture. The capacity of TN thymocytes to differentiate was exclusively confined to the $CD34^+$ population. $CD34^-$ TN thymocytes failed to differentiate in this system. In contrast, cloned lines of $CD3^-$ thymocytes could only be established from $CD34^-$ TN thymocytes. Five subsets of $CD3^-$ thymocytes were found with the following phenotype: $CD1^-$TN, $CD1^+$TN, $CD1^+CD4^+CD8^-$, $CD1^+CD4^+CD8\alpha^+\beta^-$, and $CD1^+CD4^+CD8\alpha\beta^+$. These subpopulations expressed decreasing levels of CD34. The $CD1^-CD3^-$ population expressed the highest levels of CD34 supporting the notion that this population is the most immature T cell precursor in the thymus, whereas the $CD1^+CD4^+CD8\alpha^+\beta^+$ which did not express CD34 seems to be the most mature of these $CD3^-$ populations. This notion is supported by the observations that $CD34^+$ cells isolated from fetal liver, which differentiated into T cells in a FTOC, developed into $CD3^+$ cells via $CD1^-$ and $CD4^+CD8^-$ intermediates. Based on these data, we present a model of early stages in human intrathymic development.

It is generally accepted that the most early thymic T cell progenitors are cortical thymocytes that do not express the TCR–CD3 complex or the coreceptors CD8 and CD4. In the cortex, these cells develop into thymocytes expressing CD4 and CD8 and low levels of CD3. A small proportion of these double positive (DP) cells are rescued from programed cell death by positive selection, presumably involving TCR/MHC and appropriate CD4-CD8/MHC interactions, and develop further into single positive (SP) thymocytes (1, 2). Studies of intrathymic developmental pathways in mice have been aided by the availability of an in vitro fetal organ culture system (3, 4) and intrathymic injection into host mice (5–7). These assays have been particularly useful for delineation of early events in thymic development and have allowed characterization of the most immature murine T cell precursors. By contrast, the study of human thymic T cell progenitors has been hampered by the lack of markers as well as differentiation assays that unequivocally define these cells, although there is consensus that the most immature human thymocytes lack CD3, CD4, and CD8 (8–11). Several groups have reported that human triple negative (TN)[1] cells respond to IL-2 and differentiate in vitro to TCR-α/β^+ or to TCR-γ/δ^+ cells in simple culture systems either in the presence or absence of thymic stromal cells (12–15). In contrast, we found that highly purified TN thymocytes do not respond to IL-2 or IL-4, but strong proliferation was observed in the presence of IL-7. Short-term culture of TN thymocytes in IL-7 induced CD4 and CD8α on these cells, but the cells did not acquire the CD3-TCR complex upon culture in IL-7 (16). We reasoned that differences in the methods to purify TN thymocytes and the criteria to assess purity of the TN cell samples could be responsible for the differences in results reported by different groups (16). To solve this question it was important to find a marker that would positively identify immature thymocytes and to develop an assay to mea-

[1] *Abbreviations used in this paper:* DP, double positive; FTOC, fetal thymic organ culture; lin, lineage; SP, single positive; TN, triple negative.

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sure the capacity of these cells to differentiate into $CD3^+$ cells. A potential marker for intrathymic T cell progenitors is CD34. CD34 is a 120-kD cell surface antigen that is expressed on pluripotent hematopoietic stem (17–19) cells and on precursors that are committed to several hematopoietic lineages, including the B (20), myeloid, and erythroid lineages (17, 18). CD34 is therefore not a lineage-specific marker, but can be considered as a stage-specific marker identifying immature hematopoietic cells. Although a number of groups have reported expression of CD34 in immature thymocytes, the proportion of thymic TN cells that express CD34 is controversial. Kurtzberg et al. (21) reported that 26% of these cells express CD34, while Poggi et al. (22) did not detect CD34 on $CD1^-$ TN thymocytes, considered to be the most early T cell precursors in the thymus (22). In contrast, we have found that in most samples of thymocytes that were rigorously depleted of CD3,CD4, and CD8 positive cells by a combination of magnetic and fluorescent cell sorting, >90% of the cells expressed CD34 (16). Similar findings were reported by Terstappen et al. (23). The finding that $CD34^+$ thymocytes coexpress CD2, CD5, and CD7, suggest that these cells are T cell precursors (16, 23), but direct proof of this notion is lacking. Moreover, the possibility that $CD34^-$ TN thymocytes also contain T cell precursors could not be dismissed.

It has been reported that CD34 cells from fetal liver or fetal bone marrow can develop into T cells when injected into thymic fragments that are subsequently transplanted in SCID mice (24). Jenkinson and co-workers (3, 4) have shown that in the mouse system early thymic progenitors can develop in fetal thymic lobules in vitro, indicating that an in vivo environment is not an absolute requirement for T cell development. Based on this information, we developed a human in vitro fetal thymic organ culture (FTOC) system to investigate the T cell differentiating potential of $CD34^+$ and $CD34^-$ TN thymocytes. To obtain information about the early stages of human T cell development, we examined the kinetics of development of $CD3^+$ cells from $CD34^+$ fetal liver cells.

Materials and Methods

Preparation of Thymocyte Suspensions and Thymocyte Subpopulations. Thymic tissue was obtained from children of 3 mo to 10 yr of age undergoing median sternotomy and corrective cardiovascular surgery. Suspensions were made by mincing tissue and pressing through a stainless steel mesh. Large aggregates were removed and the cells were washed once before separating subpopulations. For preparation of $CD34^+$ TN cells, thymocytes were incubated with the antibodies RPA-T4 (CD4) and RPA-T8 (CD8) (kind gifts of Dr. G. Aversa, DNAX Research Institute) and then incubated with magnetic beads coated with sheep anti-mouse antibody (Dynal, Oslo, Norway). After a first round of depletion with a magnet, the cells were incubated with PE-labeled antibodies against CD19 (Leu12), CD14 (LeuM3), CD33 (LeuM9), CD56 (Leu19) (all Leu antibodies obtained from Becton Dickinson & Co., San Jose, CA), and with an antibody against glycophorin (10F7MN; American Type Culture Collection (ATCC), Rockville, MD) followed by another round of magnetic sorting to remove B, NK, myeloid, and erythroid cells. The remaining cells were then incubated with FITC-labeled anti-CD34 (HPCA-2; Becton Dickinson & Co.) and with PE-labeled CD3 (Leu4), CD4 (Leu3a), and CD8 (Leu2a). Leu2a and Leu3a are directed against epitopes different from RPA-T4 and RPA-T8 which were used for the magnetic bead depletion. $CD34^+$, $CD3^-CD4^-CD8^-CD14^-CD19^-CD33^-CD56^-$ cells were then sorted with the FACStar Plus® (Becton Dickinson & Co.). Only cells that were more than 99% pure upon reanalysis, were used for subsequent experiments.

To prepare $CD8\beta^-$ samples, total thymocytes were incubated with anti-$CD8\beta$ antibodies (2ST8-5H7; a kind gift of Dr. E. Reinhertz, Dana Farber Cancer Institute, Boston, MA) for 30 min at 4°C, and then with magnetic beads coated with goat anti-mouse antibody. PE-labeled CD3 (Leu4, Becton Dickinson & Co.) was added to the remaining cells followed by another round of magnetic bead depletion. PE-Leu4 was used at this point to check for possible modulation of CD3 during the separation, since internalized anti-CD3-PE would still be detectable because of the brightness of the fluorochrome. To remove resident thymic B cells, monocytes, myeloid progenitors, NK cells, and erythrocytes, antibodies against CD19 (Leu12), CD14 (LeuM3), CD33 (LeuM9), CD56 (Leu19), and glycophorin (10F7MN) were also added at this stage. Remaining $CD3^+$, $CD8\beta^+$, $CD14^+$, $CD19^+$, $CD33^+$, $CD56^+$, and glycophorin$^+$ cells were stained with PE-labeled goat anti-mouse IgG or IgG2a (Caltag Laboratories, San Francisco, CA), and PE-stained cells were removed by sorting on the FACStar Plus®. For three-color analysis of the $CD3^-CD8\beta^-$ thymocytes, we used Leu2(CD8)-FITC, HPCA-2(CD34)-PE, and Leu3(CD4) labeled with peridinin chlorophyll protein (PerCP; Becton Dickinson & Co.).

Isolation of $CD34^+$ Fetal Liver Progenitors. Fetal liver tissue (18 wk of gestational age and $HLA\text{-}A2^+$) was homogenized through a wire mesh under a stream of RPMI 1640 containing 10% FCS. The cellular suspension was centrifuged over Histopaque (Sigma Chemical Co., St. Louis, MO). The remaining RBC were removed by negative selection using magnetic beads. Cells were incubated with anti-glycophorin antibody (10F7 MN; obtained from the ATCC) for 30 min at 4°C. The cells were washed twice with PBS supplemented with 5 mg/ml BSA (Sigma Chemical Co.) and 0.2 mg/ml NaN_3, and then incubated with with magnetic beads coated with purified sheep anti-mouse IgG (Dynabeads, Dynal). After removal of cells bound to beads with the magnet (Dynal), these cells were incubated for 30 min at 4°C, with saturating amounts of the following mAbs: Leu4-FITC (CD3), Leu3a-FITC (CD4), Leu2a-FITC (CD8), Leu16-FITC (CD20), Leu12-FITC (CD19), and LeuM3-FITC (CD14) (all obtained from Becton Dickinson & Co.); IOM13-FITC (CD13; AMAC, Inc., Westbrook, ME), and L185-FITC (CD56; kindly provided by Dr. J. Phillips, DNAX Research Institute). After washing, cells were incubated with Dynabeads, coated with sheep anti-mouse IgG, and the lineage $(lin)^+$ cells were removed. The depleted cell population, which still contained some FITC-labeled cells, was stained with HPCA-2-PE mAb (CD34) and the $CD34^+$ lin^- and the $CD34^-$ lin^- cells were sorted with a FACStar Plus®. Both cell populations were >99% pure upon reanalysis.

FTOC. Human fetal tissue, from donors of 17–20 wk of gestational age, was obtained from elective therapeutic abortions (Advanced Bioscience Research, Alameda, CA). Thymic fragments, containing 3–8 lobules were placed on nucleopore filters (0.8 μm; Costar Corp., Cambridge, MA) over gelfoam rafts (Upjohn, Kalamazoo, MI) and cultured for 7 d on 6-well plates (Becton Dickinson & Co.) at 25°C, 5% CO_2 in Yssel's medium (25) supplemented with 1% human serum. To avoid expansion of remaining endogenous thymocytes, the fragments were irradiated at 500 rad

before injection. Each thymic fragment was microinjected with purified CD34+ TN thymocytes with 1-mm micropipettes (World Precision Instruments, Inc., Sarasota, FL) in 0.5 μl of Yssel's medium using a Nikon injector (Nikon, Narishige, Japan). The injected thymic fragments were returned to the filters on the rafts and cultured in Yssel's medium at 37°C in a humidified 5% CO_2 atmosphere. At the end of the incubation periods, thymic pieces were homogenized by gentle pipetting in PBS containing 5 mg/ml BSA and 0.2 mg/ml NaN_3 and subjected to phenotypic analysis.

Three-color phenotypic analysis of 2–3 × 10^4 cells was performed after incubating the cells with PBS supplemented with 2% FCS and 2% normal mouse serum for 10 min, followed by 30 min staining with anti-HLA-A2-FITC mAb (CR11-351; kindly provided by Dr. S. Ferrone, New York Medical College, Valhalla, NY) or with HLA-A3-FITC (GAP-A3; ATCC) and CD1-PE (T6-RD; Coulter Corp., Hialeah, FL), and with biotinylated or PE-labeled Leu4 (CD3), Leu3a (CD4), and Leu2a (CD8). After two washes in PBS containing 5 mg/ml BSA and 0.2 mg/ml NaN_3, the cells were incubated with Streptavidin TriColor (Caltag Laboratories), washed, and subjected to cytofluorometric analysis with a FACScan® (Becton Dickinson & Co.). The results are expressed as the percentage of cells expressing a given antigen within the HLA-A2 or HLA-A3 positive population. The number of cells per piece were calculated by multiplying the total number of cells recovered per fragment of thymus by the percentage of HLA-A2 or A3+ cells in the population.

Cloning of Immature Thymocytes. TN thymocyte subsets were cloned as has been described previously (26). Briefly, dilutions of CD34+ and CD34− cells were deposited in one well of a 96-well plate (Costar Corp.) in 100 μl Yssel's medium containing a feeder cell mixture composed of 10^5 irradiated (4,000 rad) PBMC, 10^4 irradiated (5,000 rad) JY (EBV-transformed B cells) cells, and 5 ng/ml purified PHA (Wellcome, Beckenham, Kent, UK). 7 d later, 20 U/ml IL-2 (a kind gift of Dr. G. Zurawsky, DNAX Research Institute) was added, and 14 d after the onset of the cloning growing cultures were transferred into 24-well plates and restimulated with feeders. 10 d later, individual clones were analyzed for expression of CD3, CD4, CD8, and CD56.

Results

Purified CD34+ TN Thymocytes Develop into DP Thymocytes in a FTOC. HLA-A3+ thymocytes were depleted of CD4+, CD8+, B, NK, myeloid, and erythroid cells by magnetic bead depletion as indicated in Materials and Methods. PE-labeled antibodies against CD19 and CD56 were used during the magnetic bead depletion. The remaining cells were labeled with anti-CD3-PE, with PE-labeled antibodies against CD4 and CD8 (directed against different epitopes as those used for the magnetic bead depletion) and CD34-FITC. Electronic gates were placed on the FITC+PE− (CD34+) and FITC−PE− (CD34−) cells as indicated in Fig. 1 followed by sorting. Different numbers of CD34+ cells (3 × 10^2, 3 × 10^3, and 3 × 10^4), which were 99.3% pure and 3 × 10^4 CD34− cells (99.4% pure) were injected into HLA-A3− fetal thymic fragments, that were precultured for 7 d at room temperature and irradiated to remove endogenous thymocytes. The injected fetal thymic fragments were cultured at 37°C at an air-liquid interphase for 21 d. Fig. 1 shows that only the CD34+ cells developed into HLA-A3+ CD3+ cells in this FTOC. The very few cells recovered from fragments in-

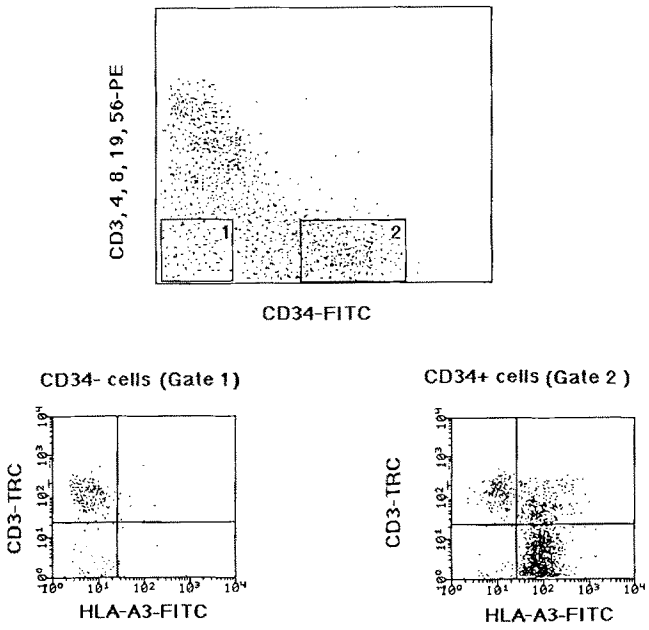

Figure 1. CD34+ thymocytes differentiate into CD3+ cells, whereas CD34− cells do not grow or differentiate into CD3+ T cells in FTOC. After depletion of CD3, CD4, CD8, CD19, and CD56 positive cells, CD34+ and CD34− cells were sorted after setting of the gates as indicated in the figure, and 3 × 10^3 CD34+ and 3 × 10^4 CD34− cells were injected into thymic fragments as indicated in Materials and Methods. The thymic fragments were cultured for 21 d as indicated in Materials and Methods. The fragments were homogenized, the recovered cells were counted and subjected to three-color analysis as indicated in Materials and Methods. The number of cells recovered after injection of 3 × 10^3 CD34+ cells was 1.3 × 10^4 cells per fragment and of 3 × 10^4 CD34− TN cells was 0.15 × 10^4 cells per fragment.

jected with CD34− thymocytes were HLA-A3− indicating their recipient origin. Fig. 2 demonstrates that 300 cells injected per fragment resulted in the appearance of CD4+CD3+ cells of donor origin. The great majority of the HLA-A3+ thymocytes that developed in the thymic organs expressed CD1 and were DP (Fig. 3), whereas ~1% of the cells are $CD3^{high}CD1^-$, indicating that some mature thymocytes of donor origin were present in these organs. A minor population of HLA-A3− (recipient derived) cells was also present in suspensions of fragments injected with different doses of purified HLA-A3+CD34+ thymocytes (Fig. 2). Fig. 3 shows that the majority of the HLA-A3− thymocytes lacked CD1 and CD8. In addition, almost all these HLA-A3− cells expressed high levels of CD3 and CD4 (Fig. 4). Taken together, these data indicate that the majority of residual recipient thymocytes that are still present in the thymic fragments after 21 d of culture are mature CD3+CD4+ cells. Although not shown, short-term incubation (up to 6 d) of CD34+ thymocytes in the presence of IL-7 alone or with thymic epithelial cells (TEC), established as described previously (27, 28), and IL-7 did not result in the generation of either TCR-γ/δ or TCR-α/β cells. The frequency of contaminating more mature T cells present in the purified CD34+ cells used to inject the thymic fragments was checked by limiting dilution under conditions that allow high

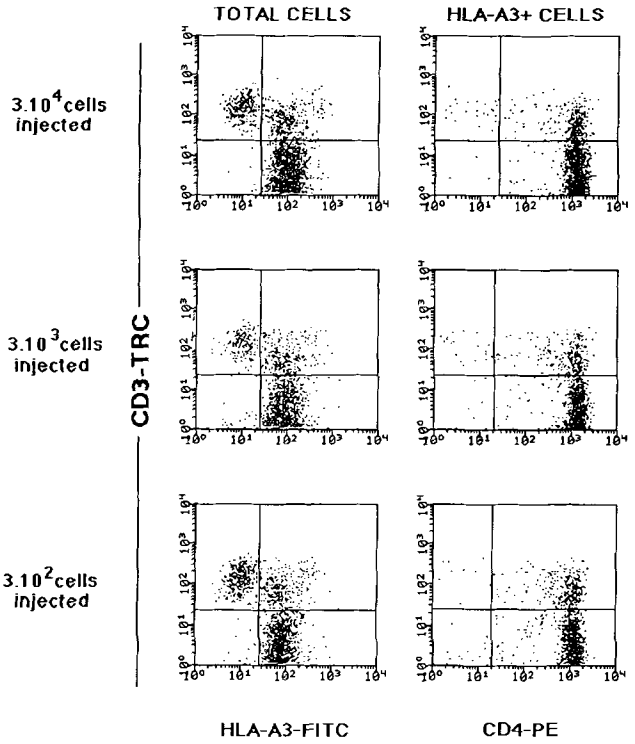

Figure 2. Development of varied numbers of HLA-A3$^+$ CD34$^+$ TN thymocytes in HLA-A3$^-$ fetal thymus organs. 3 × 10^2, 3 × 10^3, and 3 × 10^4 CD34$^+$ cells were injected into individual fetal thymic fragments and cultured for 21 d as indicated in Materials and Methods. The fragments were homogenized and the recovered cells were counted and subjected to three-color analysis as indicated in Materials and Methods. The recoveries of donor-derived (HLA-A3$^+$) cells per fragment were: after injection with 3 × 10^4 cells, 2.6 × 10^4 cells were recovered per fragment, after injection with 3 × 10^3 cells, 1.3 × 10^4 cells, and after injection with 3 × 10^2 cells, 0.4 × 10^4 cells.

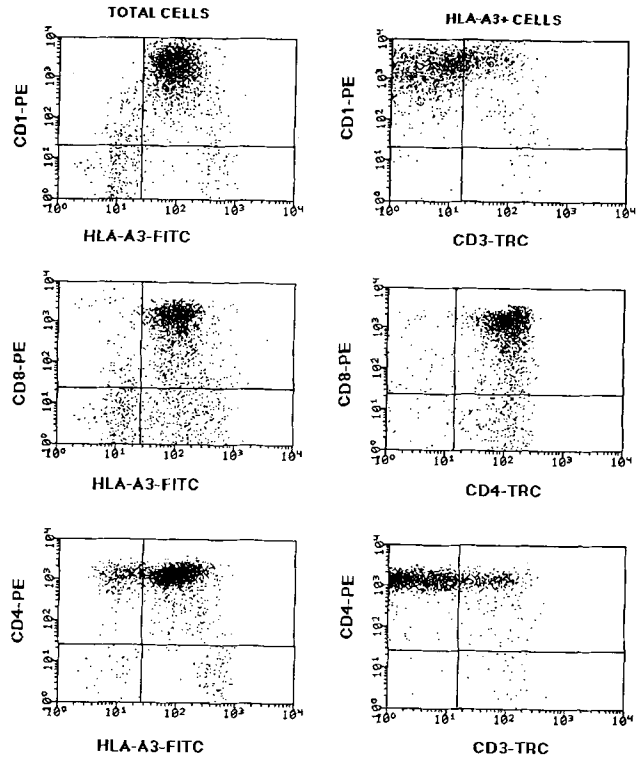

Figure 3. Expression of CD1, CD3, CD4, and CD8 on thymocytes recovered from FTOC. Cells recovered from HLA-A3$^-$ thymic fragments injected with 3 × 10^4 HLA-A3$^+$ cells per fragment were subjected to three-color analysis using labeled antibodies as indicated in Materials and Methods and analyzed on a FACScan®.

cloning efficiencies of mature T cells. The frequency of TCR/CD3$^+$ cells that were clonogenic under those conditions was <1:10^3.

Thymocyte Subpopulations that Express CD34. Previously we have shown that CD1 subdivides TN thymocytes into two subsets (16). The proportion of CD1$^+$ vs CD1$^-$ TN thymocytes varied from donor to donor, but the number of CD1$^-$ TN cells exceeded that of CD34$^-$ cells. Therefore it was expected that part of the CD1$^-$ cells would express CD34. Fig. 5, which shows a double staining of TN thymocytes with anti-CD1-PE and anti-CD34-FITC, demonstrates that essentially all CD1$^+$TN cells express CD34. A proportion of CD1$^-$ cells expressed CD34, and expression of CD34 on the CD1$^-$CD34$^+$ cells was higher than on CD1$^+$CD34$^+$ thymocytes (Fig. 5). These results strongly suggest that CD1$^-$CD34$^+$ cells are the most immature cells followed by CD1$^+$CD34$^+$ TN thymocytes. Previously we have demonstrated the existence of CD3$^-$CD4$^+$CD8$^-$ and of CD3$^-$CD4$^+$CD8$\alpha^+\beta^-$ populations and provided evidence that these subsets were the progeny of TN thymocytes (16). To confirm the immature status of these subpopulations, we analyzed CD34 expression on these cells. In pilot experiments, it was found that CD34 is not expressed on CD8β^+CD3$^+$ thymocytes as assessed with double staining using PE-labeled anti-CD34 and FITC-labeled anti-CD3 and

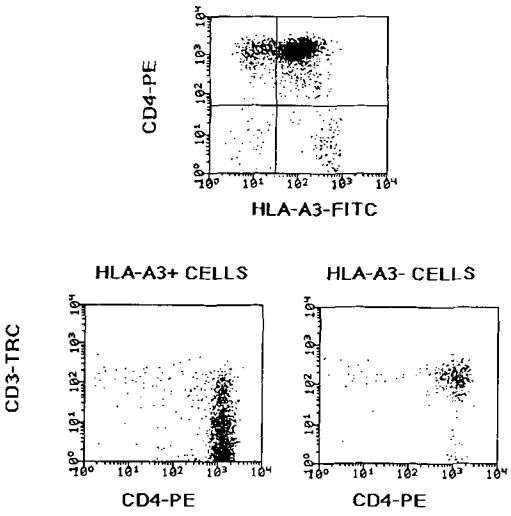

Figure 4. Expression of CD3 and CD4 on donor-derived HLA-A3$^+$ and recipient HLA-A3$^-$ cells recovered from a FTOC. HLA-A3$^-$ and HLA-A3$^+$ cells from the same sample as shown in Fig. 2, were analyzed for CD3 and CD4 expression.

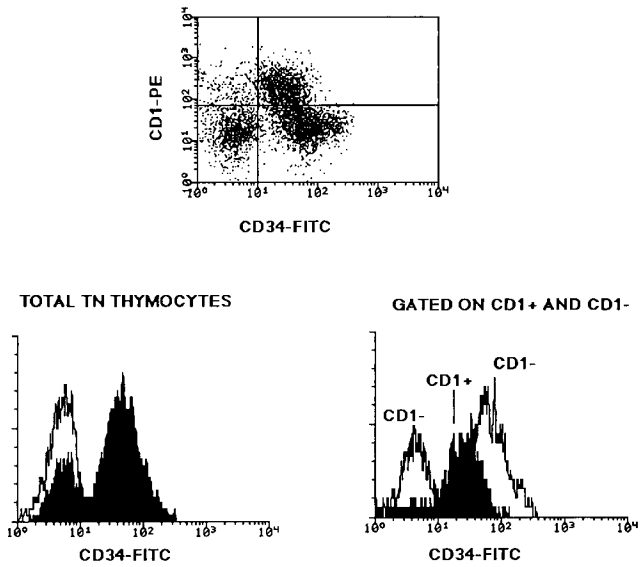

Figure 5. Expression of CD1 and CD34 on TN thymocytes. Thymocytes were depleted of CD3, CD4, and CD8 positive cells by a combination of magnetic bead depletion and sorting as indicated in Materials and Methods and analyzed for expression of CD1 and CD34. CD34 was expressed on 75% of the cells in this sample. The nature of the $CD34^-$ cells in this sample was not investigated further.

$CD8\beta$ mAbs (results not shown). Therefore we restricted our analysis of CD34 expression to thymocytes that were depleted of $CD3^+CD8\beta^+$ cells. A three-color analysis of thymocytes that were depleted of CD3 and $CD8\beta$ positive

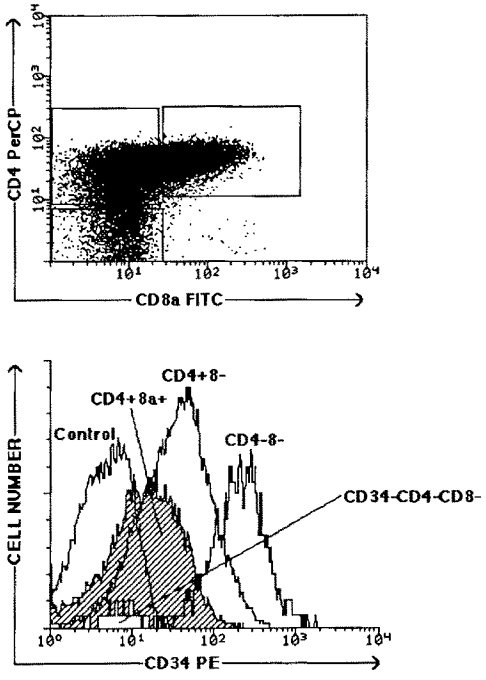

Figure 6. Expression of CD34, CD4, and CD8α on $CD3^-CD8\beta^-$ thymocytes. Thymocytes were depleted of $CD3^+$ and $CD8\beta^+$ cells as indicated in Materials and Methods. The population was 99.5% pure (PE negative) by reanalysis.

cells was carried out using anti-CD4–labeled PerCP, anti-CD8α-FITC, and anti-CD34-PE. Anti-CD34-PE was used in this experiment rather than anti-CD34-FITC, since the former is 5–10-fold more sensitive than the latter and would allow detection of low levels of CD34 expression. Fig. 6 shows the dot plot of CD4 and CD8α. As expected from our previous data, three populations can be distinguished $CD4^-CD8\alpha^-$, $CD4^+CD8\alpha^-$, and $CD4^+CD8\alpha^+$ cells. Analysis of each of these populations for CD34 expression, clearly demonstrates that $CD4^-8\alpha^-$ expresses the highest levels of this antigen followed by $CD4^+8\alpha^-$ and then by $CD4^+8\alpha^+$ cells. Note that $CD3^-CD4^+CD8^-$ cells are all $CD34^+$, whereas TN cells contain a few $CD34^-$ cells. As shown above, these TN $CD34^-$ cells do not contain cells that have the capacity to develop into $CD3^+$ cells in a FTOC. Based on the analogy with findings indicating that expression of CD34 in the myeloid/erythroid lineages decreases upon progressing maturation, our findings strongly suggest that $CD34^+CD1^-$ TN thymocytes are the most immature followed by the $CD34^+CD1^+$, $CD34^+CD4^+CD8^-$, and the $CD34^\pm CD4^+CD8\alpha^+$ thymocytes.

Early Kinetics of Development of $CD34^+$ Fetal Liver Cells into T Cells in a FTOC. To find further evidence for the progeny–product relationship of the various immature thymocyte subsets as suggested by the level of expression of CD34, we investigated the kinetics of T cell development in FTOC. It was reasoned that the early events could best be studied by following T cell development of a source of prethymic progenitor cells in a FTOC. In early embryogenesis, pluripotent hematopoietic stem cells are present in fetal liver. Since it has been shown that $CD34^+$ fetal liver cells can develop into T cells (24), we used $CD34^+$ fetal liver cells as a source of prethymic T cell progenitors. Fetal liver cells were first depleted of erythrocytes, T, B, NK, and mature myeloid cells, labeled with anti-CD34-PE and sorted with a FACStar Plus®. Fig. 7 shows that the sorted, lineage-negative, $CD34^+$ cells lack the T cell markers CD3, CD4, and CD8, the monocyte/myeloid markers CD13 and CD14, the B cell markers CD19 and CD20, and the NK cell marker CD56. These cells expressed HLA-A2 and were injected into $HLA\text{-}A2^-$ thymic fragments. At various intervals, thymic fragments were homogenized and the resulting cell suspensions were analyzed on a FACScan® for forward and side scatter parameters. An electronic gate was placed on cells with a small lymphoid size and expression of CD1, CD3, CD4, and CD8 was analyzed. The total number of $HLA\text{-}A2^+$ small lymphoid cells recovered from the thymic fragments was 3×10^4 on day 10, which decreased to 1.6×10^4 on day 14 and increased again via 2.1×10^4 on day 17 to 7×10^4 cells per fragment on day 20. Fig. 8 shows that on day 10 few of these $HLA\text{-}A2^+$ small lymphoid cells expressed CD3, CD4, or CD8. By day 14, 80% of these $HLA\text{-}A2^+$ small cells expressed CD4, 28% expressed CD8, and 2% of these cells were $CD3^+$. The number of small, $HLA\text{-}A2^+$ cells that expressed both CD4 and CD8 cells increased with further incubation and by day 20, most of the cells coexpressed CD4 and CD8 (Fig. 8). The percentage $CD3^+$ cells in the HLA-

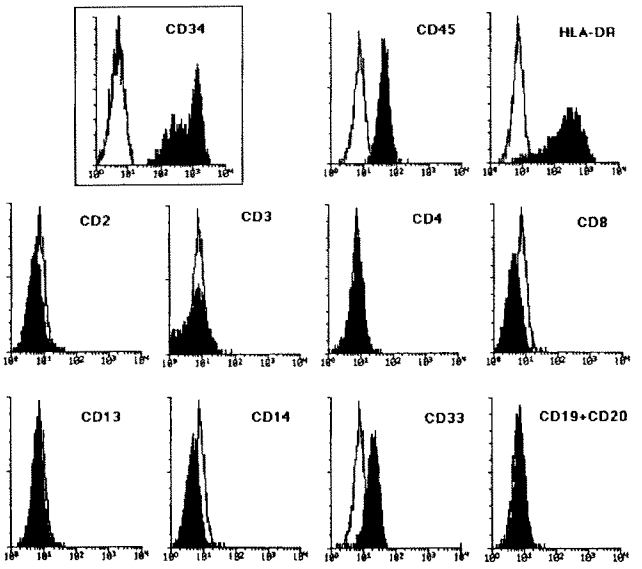

Figure 7. Phenotype of CD34$^+$ lineage$^-$ fetal liver cells. Glycophorin-negative fetal liver cells from a donor of 19 wk gestational age, were obtained as described in Materials and Methods. The cells were 99.5% pure upon reanalysis. Samples of sorted CD34$^+$ lineage negative cells were incubated with FITC-labeled antibodies for 30 min at 4°C, washed and analyzed on the FACScan®. The CD34 expression is shown in the box. Since the sorted cells were already labeled with anti-CD34-PE, we used as negative control the cells obtained after depletion with magnetic beads but before CD34 sorting, labeled with control IgG1-PE.

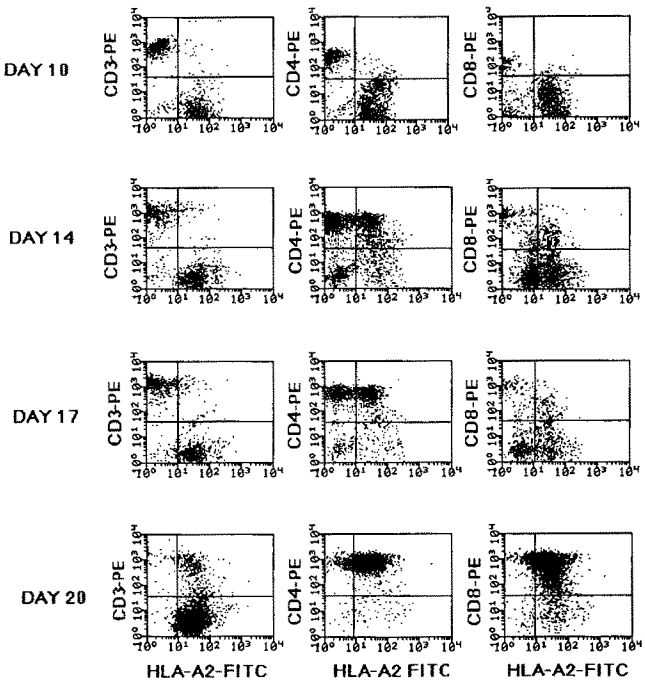

Figure 8. Early kinetics of differentiation of CD34$^+$ fetal liver cells in FTOC. CD34$^+$, lineage-negative, fetal liver cells from a donor of 19 wk gestational age, were obtained as described in Materials and Methods. The cells were 99.5% pure upon reanalysis. Analysis of lymphoid cells, developed in FTOC, was done as indicated in Materials and Methods.

A2$^+$ population increased during the culture period from undetectable on day 10 to 8% on day 20. The findings presented in Fig. 8 indicate that the thymic fragments can support differentiation of T cells, although the period of 20 d seems too short to observe complete T cell development. As expected from our phenotypic analysis, acquisition of CD4 and CD8 does not occur simultaneously. At days 14 to 17, CD4 is expressed on many more small lymphoid cells than CD8. Therefore at days 14 and 17, a considerable proportion of the cells are CD3$^-$CD4$^+$CD8$^-$. In a second experiment, we analyzed expression of CD1, CD3, CD4, and CD8 in an organ culture after incubation for a period of 15–26 d after injection of CD34$^+$ fetal liver cells. In Fig. 9 we have plotted the percentage of HLA-A2$^+$ cells that expressed CD1, CD3, CD4, and CD8 against the incubation time. It is shown that 57% of the cells express CD1 and 40% CD4 at day 15. The percentages of CD3 and CD8$^+$ cells were low (6 and 11%, respectively) at that time point (Fig. 9). This finding indicates that on day 15 the CD34$^+$ fetal liver cells have developed to CD1$^+$CD4$^-$ and CD1$^+$CD4$^+$ cells. Upon continuation of the incubation, the number of cells that expressed CD3 and CD8 increased dramatically, and by day 26 most cells expressed CD1, CD3, CD4, and CD8. These results provide further proof that the fetal thymic fragments support T cell differentiation in vitro, since fetal liver CD34 does not express CD3, CD4, or CD8 at all. Moreover, the results support the notion that CD1 appears before CD4 on the T cell progenitors, followed by CD8 and then CD3.

Clonogenic Potential of CD34$^+$ and CD34$^-$ TN Thymocytes. Previously, we and others (22, 26, 29) have reported that CD3$^-$ clones can be established from TN thymocytes. Our studies and those of Poggi et al. (22) demonstrated that many of these CD3$^-$ clones shared characteristics with NK cells. However since some of the membrane CD3$^-$ clones expressed CD3 in their cytoplasm (22, 26), the possibility that some of these clones were derived from T cell precursors, could not be ruled out. Therefore we performed several experiments in which we cloned purified CD34$^+$ and CD34$^-$ TN thymocytes under conditions that have been used previously by us and by Denning et al. (29) to isolate CD3$^-$

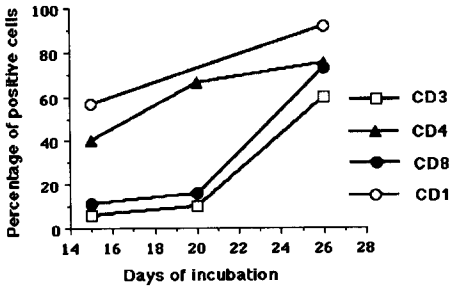

Figure 9. Kinetics of appearance of CD1, CD3, CD4, and CD8 on CD34 fetal liver cells. CD34$^+$, lineage-negative cells of a donor of 18 wk gestational age were purified and injected into HLA-A2$^-$ thymic fragments. Plotted is the expression of CD1, CD3, CD4, and CD8 on small lymphoid HLA-A2$^+$ cells against the time.

clones from TN thymocytes. As it is summarized in Table 1, $CD3^-$ clones could only be isolated from $CD34^-$ TN cells that are not capable of developing into $CD3^+$ thymocytes in a FTOC. The $CD34^+$ TN thymocytes that develop into $CD3^+$ thymocytes in FTOC do not give rise to $CD3^-$ clones. In experiment 1, TN cells were cloned that included $CD34^+$ and $CD34^-$ cells and that were not depleted of $CD56^+$ cells. With the exception of one $CD3^-$ clone, all clones isolated in experiment 1, expressed CD56 and were cytotoxic for the NK-sensitive target cell K562 (percent ^{51}Cr-release >30% at an E/T ratio of 5:1). One $CD3^-$ clone did not express CD56 and was not cytotoxic for K562 (5% ^{51}Cr-release at an E/T ratio of 5:1). $CD3^+$ clones were isolated after cloning of $CD34^+$ cells in experiment 2, but the frequency of these clones (1/1,000) and the fact that the $CD34^+$ cells used in this experiment were <99% pure, make it likely that the $CD3^+$ clones isolated in experiment 2 were derived from contaminating cells. This notion is supported by the results of experiments 3, 4, and 5, which demonstrate that cloning of $CD34^+$ TN cells do not yield clones at all (experiments 4 and 5) or clones with an extremely low frequency (experiment 3). In contrast, cloning of $CD34^-$ cells yielded $CD3^-$ clones, although the frequencies of those clones was not high (1/330 and 1/840 in experiments 4 and 5, respectively). The 22 $CD3^-$ clones isolated in experiment 5 were tested for expression of CD56 and cytotoxic activity against K562. 13 clones did not express CD56 and did not kill (<15% lysis in a ^{51}Cr-release assay) K562 in vitro. Nine clones expressed intermediate or high levels of CD56 and had various levels of cytotoxicity against K562 (20–95% ^{51}Cr-release at an E/T ratio of 5:1) and can be considered to be NK cells. These experiments support the conclusion that the $CD34^+$ cells are not clonogeneic under our in vitro conditions.

Discussion

Using a novel human FTOC system, we demonstrate in this report that $CD34^+$ TN thymocytes are able to develop into $CD4^+CD8^+CD3^+$ thymocytes, whereas $CD34^-$ TN thymocytes lack this capacity. We have shown in a representative experiment (out of three) that 300 $CD34^+$ thymocytes that were >99% pure upon reanalysis develop into $CD3^+$ thymocytes in the FTOC. It is highly unlikely that the $CD3^+$ cells observed in the FTOC would have been exclusively derived from those $CD3^+$ cells contaminating the injected $CD34^+$ cells. More importantly, $CD34^-$ cells, sorted from the same samples, which might have similar contaminations with $CD3^+$ cells, did not develop to $CD3^+$ thymocytes. Further evidence for the notion that the fetal thymic fragments induce T cell differentiation in vitro is provided by the fact that $CD34^+$ fetal liver cells rigorously depleted of any CD3, CD4, or CD8 positive cells develop into $CD1^+CD3^+CD4^+CD8^+$ cells in the FTOC.

Differentiation of CD34 TN thymocytes into $CD3^+$ cells has been investigated previously (21). These authors reported that both $CD34^+$ and $CD34^-$ TN thymocytes can develop into $CD4^-CD8^-TCR\text{-}\gamma/\delta^+$ cells after coculture with TEC and IL-2. $TCR\text{-}\alpha/\beta^+$ cells were not found in those cultures. In our hands (16) TN thymocytes as well as purified $CD34^+$ thymocytes (data not shown) did not respond to IL-2 and no clear evidence was found for differentiation of $CD34^+$

Table 1. *Cloning of $CD34^+$ and $CD34^-$ TN Thymocytes*

Expt.	Cell source	Purity	Cloning efficiency	No. of clones tested	$CD3^+$ clones	$CD3^-CD56^+$ clones	$CD3^-CD56^-$ clones
		%					
1	TN*	99.5	1/110‡	39	2	36	1
2	$CD34^+$§	98.7	1/1,000	16	16	0	0
3	$CD34^+$	99.7	1/10,000	6	2	4	ND
4	$CD34^+$	99.8	0	0	–	–	–
	$CD34^-$§	99.7	1/330	39	0	39‖ ($CD3^-$ only)	ND
5	$CD34^+$	99.8	0	0	–	–	–
	$CD34^-$	99.1	1/840	29	5	9	13

* TN thymocytes were obtained after depletion of CD3, CD4, CD8 positive cells by magnetic bead and FACS® sorting. Those cells were not further purified and contained 1.5% $CD56^+$ cells.

‡ Cloning efficiencies were calculated based on limiting dilution. Graded numbers, usually 1, 10, 100, and 1,000 cells per well, were cultured in wells of a 96-well plate with feeder cells as indicated in Materials and Methods. Fractions of negative wells were plotted against the number of seeded cells. The cells containing one clonogenic cell were determined by the dose corresponding to 0.37 negative cultures as described (28).

§ In expts. 2–5, we used purified $CD34^+$ cells that were rigorously depleted of CD3, CD4, CD8, CD14, CD19, CD33, and CD56 positive cells, as indicated in Materials and Methods.

‖ In expt. 4, expression of CD56 on the $CD3^-$ clones was not investigated.

thymocytes into TCR-γ/δ^+ after a short-term (up to 6 d) incubation in the presence of purified TEC, isolated as described previously (27, 28) and IL-7. A fundamental difference with the results of Kurtzberg et al. (21) is the fact that in our hands, $CD34^-$ cells did not possess the capacity to develop into $CD3^+$ cells either in FTOC as shown here, or in coculture with TEC and cytokines (results not shown). A possible explanation for the differences in results of our group and of Kurtzberg et al. (21) may be the purity of the cell preparations. Kurtzberg and co-workers (14, 21) depleted thymocytes from $CD3^+$ cells using complement mediated lysis and panning. In our hands, complement mediated lysis in combination with panning is inadequate to achieve a stringent purity. In addition, the possibility exists that the complement mediated lysis procedure involves incubation of TN cells with anti-CD3 mAbs at 37°C, which may lead to rapid downregulation of the TCR–CD3 complex. The depleted cell population may therefore contain phenotypically $CD3^-$ cells that are in fact mature T cells with a modulated receptor. In addition, it is possible that, in the absence of stringent fluorescent cell sorting, the $CD34^-$ populations may have been contaminated with cells that have a very low level of expression of CD3-TCR-γ/δ complex (15) which is upregulated by in vitro culture. In that case, it is appropriate to speak of maturation rather than differentiation (15). We took special care to diminish the possibility of isolating thymocytes with a low expression of CD3 or with a modulated CD3-TCR complex. The separation procedure was carried out at 4°C and crosslinking of the CD3-TCR complex during the separation procedure was avoided. Moreover, we used anti-CD3-PE, which is more sensitive than FITC-labeled anti-CD3 mAb and which allows for a more stringent sorting of $CD3^-$ cells.

Having established that thymic precursors of $CD3^+CD4^+CD8^+$ thymocytes express CD34, we were able to address the question of how different immature thymic subpopulations, which have been described previously, relate to one another. Here and in our previous report (16) we identified five subsets of $CD3^-$ thymocytes. These populations had different levels of CD34 expression. Terstappen et al. (30) have shown that the appearance of lineage-specific markers on several hematopoietic lineages is accompanied by a decrease in CD34 expression. For example, an increase of CD71 in the erythroid, CD33 in the myeloid lineage, and CD10 in the B cell lineage parallels a decrease in CD34 expression (30). Moreover, the level of CD34 expression in the myeloid lineage correlates with the plating efficiency of single cells for CFU blasts (30). Based on the relative expression of CD34, the results suggest that $CD1^-CD34^+$ TN thymocytes are the most immature thymocytes, as has been proposed earlier (8), and that $CD1^+CD4^+CD8\alpha^+\beta^+$ are the most mature $CD3^-$ thymocytes. The proposed sequence for early thymocyte differentiation, shown in Fig. 10, is consistent with the kinetics of development of $CD34^+$ fetal liver cells in FTOC. CD4 appears before CD8 as indicated by the observation that $CD4^+CD8^-$ lymphoid cells are present by days 14–19, whereas at later stages almost all thymocytes express both CD4 and CD8. At early time points the number of $CD1^+$ cells exceeded that of $CD4^+$ cells indicating that CD1 appears before CD4. We have not yet been able to time the appearance of $CD3^-CD4^+CD8\alpha^+\beta^-$ cells in the FTOC, because this required a four-color analysis, which is difficult to perform in a reliable fashion with the limited number of cells that is obtained in FTOC experiments. It was found however, that freshly purified $CD3^-CD4^+CD8^-$ thymocytes respond to IL-7 and develop into $CD3^-CD4^+CD8\alpha^+\beta^-$ after culture in IL-7 (data not shown), providing additional support for the notion that $CD3^-CD4^+CD8^-$ are the direct precursors of $CD4^+CD8\alpha^+\beta^-$ thymocytes.

Expression of CD4 and CD8 on immature human thymocytes is similar to that on the murine thymus, although there are some differences. A $CD3^-CD4^+CD8^-$ thymic precursor population has also been described in the mouse (31, 32). In addition to these $CD4^+$ immature cells, $CD3^+CD4^-CD8^+$ immature mouse thymocytes have been found that express both $CD8\alpha$ and β (33). The frequencies of $CD3^-CD4^+$ and $CD3^-CD8^+$ SP thymocytes seem to be strain dependent. A $CD3^-CD4^-CD8^+$ intermediate cell population has also been observed in the rat thymus (34). There is evidence that SP immature thymocytes are intermediates between TN and DP thymocytes (35, 36). Whereas our findings support the notion that $CD3^-CD4^+CD8^-$ cells are the precursors of DP thymocytes, we have not found evidence for the existence of a $CD3^-CD4^-CD8^+$ intermediate population in the human thymus, that had been postulated (37).

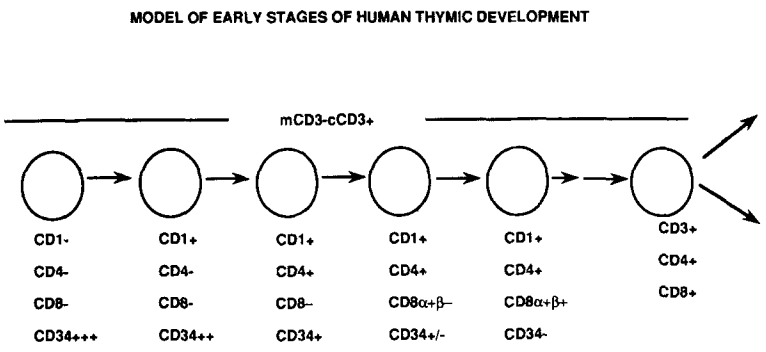

Figure 10. Model of early stages of human thymic T cell development.

Recently we and others (22, 26, 29, 38) have isolated cloned lines of $CD3^-$ cells that were derived from TN thymocytes. In our previous (26) and present study, almost all of these TN clones (derived from TN thymocytes without depletion of $CD56^+$ cells and further purification of $CD34^+$ cells) expressed CD56 and these clones mediated cytotoxic activity against NK-sensitive target cells. It was found however, in our previous study, that some of the cell surface $CD3^-$ clones expressed cytoplasmic CD3δ and ϵ proteins that were considered to be exclusively expressed in T cells, although the TCR genes of those cytoplasmic $CD3^+$ clones were in their germline configuration. Cytoplasmic CD3 proteins were also detected in thymic cell surface $CD3^-$ clones isolated by Mingari and co-workers (22, 38). Denning et al. (29) reported isolation of $CD4^+$ and $CD8^+$, SP $CD3^-$ clones from TN thymocytes using the same conditions we have used. The phenotype of the $CD4^+$ SP $CD3^-$ clones, which we have also isolated from fetal TN thymocytes (Spits, H., unpublished observations) is similar to that of the immature $CD4^+$ thymocytes, and this may have suggested that some T cell progenitors have in vitro clonogenic potential. The findings reported here, however, argue against the interpretation that $CD3^-$ clones isolated from TN thymocytes are derived from T cell precursors. The in vitro clonogenic potential of TN thymocytes resides exclusively in the $CD34^-$ TN population that has no capacity to differentiate into $CD3^+$ thymocytes. In addition, three attempts to directly clone $CD3^-CD4^+CD8^-CD34^+$ thymocytes with the FACStar Plus® or by limiting dilution failed (data not shown), indicating that the $CD3^-CD4^+CD8^-$ clones obtained in our studies and those of Denning et al. (29) are not derived from the CD4 SP precursor population. The finding that $CD34^+$ TN thymocytes do not yield in vitro clonogenic cells, does not mean that these cells do not contain NK cell precursors, as has been found in immature fetal mouse thymocytes (39). It may well be possible that $CD34^+$ cells do not differentiate to NK cells in feeder cells plus IL-2. It was observed that a number of $CD3^-$ clones obtained from $CD34^+$ TN cells (depleted of $CD56^+$ cells) did not express CD56 and were not cytotoxic. This observation strongly suggests that the majority of the $CD3^-$ clones obtained from the $CD34^-$ thymocytes are derived from $CD3^-CD56^-$ cells as we have never observed disappearance of CD56 from $CD56^+$ clones. The $CD56^+$ clones established from $CD34^-CD56^-$ thymocytes may either have been derived from contaminating $CD56^+$ cells or may have acquired CD56 during the cloning procedure. The phenotype of the $CD3^-CD56^-$ clones is very similar to that of $CD3^-CD56^-$ clones we have established from fetal liver and characterized in detail (40). The exact nature of the $CD3^-CD56^-$ cells remains unclear, but it is possible that these cells are the immediate precursors of $CD3^-CD56^+$ NK cells. Extensive characterization of $CD3^-$ $CD56^-$ clones is presently under way (Sanchez, M.-J., H. Spits, S. Verma, L. Lanier, and J. H. Phillips, manuscript in preparation). Since the $CD3^-CD56^-$ clones are derived from $CD34^-$ thymocytes that do not have the capacity to differentiate to T cells, it is unlikely that these $CD3^-CD56^-$ clones are representative of precursors of $CD3^+CD4^+CD8^+$ thymocytes. Taken together, we believe that cell surface $CD3^-$ clones derived from TN thymocytes, even those that express cytoplasmic CD3, CD4, or CD8, are more related to NK than to T cells. Expression of CD8 on NK cells is well-established. Expression of cytoplasmic CD3 is not exclusively confined to the T cell lineage but these proteins are also expressed in the majority of fetal NK cells (41).

The demonstration that all committed TN thymic T cell precursors express CD34, will allow for a better assessment of the purity of T cell progenitors after purification and will lead to a better characterization of these cells. Moreover, the finding that both $CD34^+$ fetal liver cells and $CD34^+$ T cell progenitors from thymus develop into T cells in a FTOC, will allow dissection of prethymic stages of T cell development.

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