Identification of a Common T/Natural Killer Cell Progenitor in Human Fetal Thymus

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Summary

The phenotypic similarities between natural killer (NK) and T cells have led to the hypothesis that these distinctive lymphocyte subsets may be developmentally related and thus may share a common progenitor (Lanier, L. L., H. Spits, and J. H. Phillips. 1992. Immunol. Today. 13:392; Rodewald, H.-R., P. Moingeon, J. L. Lurich, C. Dosiou, P. Lopez, and E. L. Reinherz. 1992. Cell. 69:139). In this report, we have investigated the potential of human CD34+ triple negative thymocytes ([TN] CD3-, CD4-, CD8-) to generate both T cells and NK cells in murine fetal thymic organ cultures (mFTOC) and in vitro clonogenic assays. CD34+ TN thymocytes, the majority of which express prominent cytoplasmic CD3e (cytoCD3e) protein, can be divided into high (CD34bright) and low (CD34dim) surface expressing populations. CD34bright TN thymocytes were capable of differentiating into T and NK cells when transferred into mFTOC, and demonstrated high NK cell clonogenic capabilities when cultured in interleukin (IL)-2, IL-7, and stem cell factor (SCF). Likewise, CD34bright TN thymocyte clones after 5 d in culture were capable of generating NK and T cells when transferred into mFTOC but demonstrated clonogenic NK cell differentiation capabilities when maintained in culture with IL-2. CD34dim TN thymocytes, however, possessed only T cell differentiation capabilities in mFTOC but were not expandable in clonogenic conditions containing IL-2, IL-7, and SCF. No significant differentiation of other cell lineage was detected in either mFTOC or in clonogenic assays from CD34+ TN thymocytes. These results represent the first definitive evidence of a common T/NK cell progenitor in the human fetal thymus and delineate the point in thymocyte differentiation where T and NK cells diverge.

NK cells are a subset of lymphocytes distinct from T and B cells. Although NK cells do not express a CD3/TCR complex or rearrange the TCR genes, they share several characteristics with T cells including the expression of cytoplasmic CD3 proteins and numerous cell surface antigens (1, 3). These similarities have led to the hypothesis that NK cells may be related to T cells and thus may share a common progenitor. Indeed, it has been shown that murine triple negative (TN)1 thymocytes can give rise to T, as well as NK cells, in vivo (2, 4). However, the identification of a common T/NK cell progenitor remains elusive, since these studies did not employ clonal cell differentiation assays.

Thymic T cell progenitors capable of reconstituting thymic organ cultures are contained within the TN thymocyte population (5). Recently, we have demonstrated that human fetal TN thymocytes can be subdivided into two distinct subsets: CD34+ TN thymocytes which proliferate and generate NK cells in the cloning conditions optimal for the growth of mature NK and T cells but that were unable to reconstitute T cell differentiation in murine fetal thymic organ cultures (mFTOC); and CD34+ TN thymocytes which would not generate NK cells in cloning conditions but readily reconstituted T cell differentiation in mFTOC (6). The presence of mature NK cells and committed NK cell precursors in the fetal thymus suggested that a certain degree of NK cell differentiation may occur in the thymus from progenitors that can give rise to both T and NK cell lineages. In this study, we have examined whether human fetal CD34+ TN thymocytes contain a bipotential progenitor population capable of both T and NK cell differentiation.

Materials and Methods

Cell Preparations. Fetal thymic and liver tissue were obtained from elective therapeutic abortions and used with the approval of the Ethical Committees of our Institute and Advanced Bioscience Resources, Inc. (Alameda, CA) from which the tissues were obtained. Gestation age was determined by crown ramp length and...
ranged from 18 to 24 wk. Mononuclear cells were prepared from fetal liver by standard Ficoll-Hypaque procedures, followed by magnetic bead depletion of erythroblasts (glycophorin A positive cells), as previously described (7). Thymocyte cell suspensions were prepared as previously described (6).

mAb mAbs against the following antigens were used: CD1a (T6; Coulter, Hialeah, FL); CD2 (Leu-5b); CD3 (Leu-4, UCHT1); CD4 (Leu-3a+b, L77, A-40); CD5 (Leu-1, UCHT2); CD7 (Leu-9); CD8 (Leu-2a, RPA-T8); CD13 (Leu-M7); CD14 (Leu-M3, 63D3); CD16 (Leu-11a); CD19 (Leu-12, B43); CD28 (L293); CD33 (Leu-M9); CD34 (HPCA-2); CD38 (Leu-17); CD45 (HLE, H130); CD45RA (Leu-18); CD56 (Leu-19 and L185); and glycophorin A (10F7Mn; American Type Culture Collection, Gaithersburg, MD). mAbs were generously provided by Becton Dickinson Immunocytometry System (San Jose, CA) or purchased from Pharmingen (San Diego, CA) unless otherwise indicated.

**Immunofluorescence, Flow Cytometry, and Cell Sorting.** Methods of immunofluorescence staining and cell sorting have been described previously (6–8). Enrichment of selected thymic populations was performed by incubation with specific mAb followed by magnetic bead depletion (Dynal, Inc., Oslo, Norway), as previously described (6, 9). Three-color immunofluorescence was performed with PE, FITC, and Cy-chrome™ (Pharmingen) fluorochrome-conjugated mAbs, followed by flow cytometric analysis. Appropriate fluorochrome-conjugated isotype-matched control Igs were used in all experiments. For cell sorting, thymocytes were depleted of CD45R (CD45), CD8, and glycophorin A cells by magnetic bead treatment. Three-color sorting was performed by excluding Cy-chrome™ positive cells. Cy-chrome™-conjugated anti-CD3, -CD4, -CD8, -CD14, and -CD19 mAbs were used in all cell sorting experiments to prepare TN thymocytes.

For detection of cytoplasmic CD3, viable cells were first surface stained with Cy-chrome™-labeled anti-CD45 and PE-labeled anti-CD3 mAbs, fixed, and then incubated with FITC-labeled anti-CD3e mAb, as previously described (3). Leu 4 mAb was used to detect cytoplasmic CD3 proteins (7). The Leu 4 mAb recognizes CD3e protein only when associated with CD3γ or CD3δ proteins (3, 10).

**Cloning of TN Thymic Populations.** TN thymocytes were cloned at one cell per well in Terasaki plates (Costar Corp., Cambridge, MA) using a single cell deposition system of the FACStar Plus® flow cytometer (Becton Dickinson). Recombinant human IL-7 (100 ng/ml) and human e-kt ligand (20 ng/ml) were purchased from R&D Systems (Minneapolis, MN), and recombinant human IL-2 (100 U/ml) was produced at DNAX Research Institute (kindly provided by Dr. Zarawska). Clones were established using Yssel's experiments to prepare TN thymocytes.

Clones were established using Yssel’s experiments to prepare TN thymocytes. The human-mouse FTOC system was used as previously described (6, 15). Deoxyguanosine (dGu) (Sigma Chemical Co., St. Louis, MO)-treated mouse thymic lobes were cocultured with sorted human thymic TN cell populations (1,000 cells per lobe) for 10–20 d in IMDM ( Gibco BRL, Gaithersburg, MD), containing 2% human AB serum. After culture, thymic lobes were homogenized in PBS and phenotypic analysis was performed. Cy-chrome™-conjugated anti-human CD45 mAb was used to identify human cells. In some experiments, fetal murine thymic lobes were reconstituted with cells from pools of CD34bright TN thymocyte-derived clones as described in the previous section. These particular mFTOC were cultured for 10 d, homogenized, and then maintained in suspension culture for an additional 7 d in IL-2 stimulation conditions.

**Results**

**NK Cells Can Be Generated in a Thymic Environment from CD34+ Thymocyte Progenitors.** TN thymocytes can be divided into two subsets based on the expression of the hematopoietic progenitor antigen, CD34. As we have recently reported, CD34+ TN thymocytes are composed primarily of mature NK cells and NK cell committed precursors, whereas CD34− TN thymocytes contain transplantable T cell progenitors (6). Although CD34+ TN thymocytes can readily differentiate to T cells in mFTOC, we also observed the appearance of a small percentage (2–6%) of mature NK cells (CD56+, CD5−, surface CD3−) in mFTOC reconstituted with CD34+ TN thymocytes (Fig. 1). NK cells present in mFTOC demonstrated antigenic expression similar to NK cells present in freshly isolated fetal TN thymocytes (data not shown). Since we have previously demonstrated that thymic NK cells and committed NK cell precursors were unable to expand in mFTOC (6), the appearance of mature NK cells in these mFTOC suggested that a progenitor for NK cells was contained within the CD34+ TN thymocytes.

**Antigenic Phenotype of Fetal TN Thymocytes.** To identify a possible NK cell progenitor, the CD34+ TN thymocyte population was analyzed by three-color flow cytometry. As displayed in Fig. 1, fetal TN thymocytes demonstrated a trimodal distribution of CD34 expression. The majority of TN thymocytes expressed low surface density CD34 (CD34dim), while coexpressing high surface density CD5, CD2, CD7, CD28, and CD38 antigens (Fig. 2A). These CD34dim thymocytes also expressed CD1, an antigen associated with thymic T cell differentiation (5, 16). The antigenic phenotype of CD34dim TN thymocytes was thus consistent with the hypothesis that these cells represented thymic T cell committed progenitors. TN thymocytes also contained a small percentage (7–18%) of cells expressing high surface density CD34 (CD34bright). Unlike the CD34dim thymocytes, the CD34bright subset displayed a more immature antigenic phenotype with variable expression of CD5, CD2, CD28,
CD38, and HLA-DR. The CD34^bright TN thymocytes, however, expressed high surface density CD7 (an antigen associated with T and NK cell lineages) (1, 17, 18), and surprisingly, also expressed surface CD13 and CD33 antigens (Fig. 2A). It is generally believed that the expression of CD13 and CD33 on progenitor populations is indicative of myeloid/monocytic lineage commitment (19, 20).

The expression of CD13 and CD33 antigens on CD34^bright TN thymocytes raised the possibility that these thymocytes may represent a multipotential hematopoietic stem cell population (16, 21-23). To address this question, TN thymocytes were analyzed for the expression of cytoplasmic CD3e (cytoCD3e) protein. CytoCD3e expression is unequivocally associated with T and NK cell lineages. To determine the capacity of various CD34^+ TN thymocyte populations to generate T and NK cells in mFTOC, TN thymocytes were subdivided according to the expression of CD34 and CD5 antigens (Fig. 3A). Three populations of TN thymocytes (I-CD34^dim, CD5^+; II-CD34^bright, CD5^+; and III-CD34^bright, CD5^-) were sorted to >96% purity and transferred into mFTOC. After 14-20 d in culture, the lobes were dissociated and analyzed for the expression of the T-cell-associated antigens, CD3, CD5, CD4, and CD8 and the appearance of mature NK cells. CD34^+ TN thymocytes were efficient progenitors for T cells in mFTOC (Fig. 3). The majority of cells in these lobes expressed surface CD5, CD4, CD8 (>95%), and CD3 (60-70%), antigens characteristic of normal T cell development (5, 25). In most experiments, NK cells were undetectable in mFTOC reconstituted with CD34^dim, CD5^+ progenitors (Fig. 3, D-G). Phenotypically mature NK cells represented 8-33% of the human CD45^+ cells in mFTOC reconstituted with CD34^bright TN progenitors. NK cells developing in these mFTOC were indistinguishable from endogenous thymic NK cells, substantiating the hypothesis that the thymic microenvironment can support the differentiation of NK cells. Hematopoietic cells of other lineages, such as B cells (CD19^+), or monocytes (CD14^-), were not observed in these reconstituted mFTOC.

Clonogenic Potential of CD34^+ Thymocytes. Although CD34^bright TN thymocytes can readily generate T cells and NK cells in mFTOC, these experiments were not performed using single cell reconstitution. Since transfer of 1,000 cells was required for consistent reconstitution, it can be argued that the CD34^bright subset may contain unique T and NK cell committed progenitors and not a single bipotential progenitor. To address this question, we developed optimal in vitro single cell cloning conditions for CD34^bright TN thymocytes using IL-2, IL-7, and SCF as growth factors. Cloning efficiencies of 70-95% were consistently observed (Table 1). It is interesting that these cloning conditions did not support the clonal expansion of CD34^dim TN thymocytes, thymic T cells, thymic NK cells, or mature peripheral blood NK cells.
Cloning conditions which we have previously shown to be optimal for the cloning of mature NK and T cells (11) were also unable to support the clonal expansion of CD34+ TN thymocytes. After 3 mo in culture, clones derived from the CD34Bright, CD5+, and the CD34Bright, CD5- subsets had expanded sufficiently to allow for analysis of function, antigenic phenotype and TCR gene rearrangement. All clones derived from the CD34Bright TN thymocyte subset displayed a typical NK cell antigenic phenotype, with high surface density CD56 expression and no detectable surface CD3 or CD5 antigens (Fig. 4, C and E). Consistent with their antigenic phenotype, these clones demonstrated strong cytolytic activity against NK-sensitive targets, K562 and Jurkat (Fig. 4, D and F). Since NK cells are also defined by their lack of TCR gene rearrangements, 100 individual clones derived from CD34Bright TN thymocytes were combined into 10 pools and analyzed.

**Figure 2.** Antigenic phenotype of fetal CD34+ TN thymocytes. (A) Freshly isolated fetal TN thymocytes were stained with Cy-chrome-conjugated anti-CD45 mAb, FITC-conjugated anti-CD34 mAb, and PE-conjugated mAbs against various leukocyte antigens. (B) TN thymocytes and T cell–depleted fetal liver cells were analyzed for the coexpression of surface CD34 and cytoCD3e antigens on CD45+ gated cells.
Figure 3. Antigenic phenotype of human thymocytes recovered from mFTOC. TN thymocytes were stained with FITC-conjugated anti-CD34 and PE-conjugated anti-CD5 mAbs and sorted (>97% purity) into three subsets: I-CD34 Dim, CD5⁺; II-CD34bright, CD5⁺; and III-CD34bright, CD5⁻.

(A) Each sorted subset was cultured in mFTOC for 15 d. (B and C) Expression of CD56, CD5, CD4, and CD8 on cells recovered from mFTOC reconstituted with I-CD34 Dim, CD5⁺ TN thymocytes. Approximately 98% of the cells expressed CD5, whereas <2% were CD5⁺, CD5⁻ NK cells. (D and E) Expression of CD56, CD5, CD4, and CD8 on cells recovered from mFTOC reconstituted with II-CD34bright, CD5⁺ TN thymocytes. 25% of the cells in these mFTOC were phenotypically CD56⁺, CD5⁻ NK cells. (F and G) Expression of CD56, CD5, CD4, and CD8 on cells recovered from mFTOC reconstituted with III-CD34bright, CD5⁻ TN thymocytes. 29% of the cells in these mFTOC were phenotypically CD56⁺, CD5⁻ NK cells. These results at day 15 are representative of four different experiments in which 8–33% of the CD45⁺ cells in mFTOC reconstituted with CD34bright thymocytes were NK cells. Similar percentages of NK cells were obtained from lobes seeded with CD34bright cells regardless of expression of CD5.

Table 1. Frequency of Clones Obtained from Thymic Populations

| Cell population | Cloning efficiency | Irradiated PBMC + JY + IL-2 + IL-7 + SCF | Irradiated PBMC + JY + IL-2 + PHA |
|-----------------|--------------------|------------------------------------------|---------------------------------|
| CD34 + 5⁻       | 70–95 (90)*        | 0                                        | 0                               |
| CD34 + 5 +      | 70–95 (88)         | 0                                        | 0                               |
| CD34 + 5 +      | 0                  | 0                                        | 0                               |

TN thymocytes were cloned as indicated in Fig. 4.

* Cultures supplemented with indicated factors (refeed weekly) were scored for wells containing >1,000 cells over a 4-wk culture period. Phenotypic and functional analysis were performed after 3 mo in culture. All clones displayed a typical NK cell antigenic phenotype (CD56⁺CD3⁻). Values represent the ranges and means (in parentheses) of three to six experiments.

by Southern blot for TCR gene rearrangements. No evidence of TCR CB or Jy rearrangement was observed in any of these clones (data not shown). Likewise, these NK clones did not differentiate into T cells when transferred into mFTOC (data not shown).

Generation of NK and T Cells from Bipotential CD34bright TN Thymocytes Clones. Although CD34bright TN thymocytes displayed high NK cell clonogenic frequencies, it can be argued that the progenitors responsible for reconstituting T cell differentiation in mFTOC were contained in the 5–30% of the CD34 Bright TN thymocytes that did not generate NK clones in clonogenic assays. To directly analyze the bipotential differentiation capacity of CD34bright TN thymocytes, CD34bright TN thymocytes were cloned in IL-7, SCF, and an irradiated melanoma cell line, MM170 as described in Materials and Methods. In preliminary experiments, these conditions were shown to induce maximum proliferation in short-term cultures, while delaying commitment to the NK cell lineage. After 5 d in culture, ~25 cells from each of the most rapidly growing clones were pooled and used to reconstitute a single fetal murine thymic organ. Since it was necessary to pool clones in order to obtain enough cells to reconstitute the mFTOC (~1,000 cells per lobe), it was critical that 100% of the clones employed in reconstituting the fetal thymic lobes were expandable and showed NK cell differentiation capacity. As displayed in Fig. 5 A, 100% of the clones demonstrated a typical NK cell antigenic phenotype, with high surface density CD56 and no detectable surface CD3 or CD5. The mFTOC reconstituted with the clones were cultured for 10 d, homogenized into single cell suspensions, and cultured an additional 7 d in IL-2 stimulation conditions (optimal for expansion of mature T and NK cells). Although the T cell reconstitution capabilities of short-term cultured CD34bright TN thymocyte clones was relatively poor, antigenic phenotype of these cultures clearly demonstrated the appearance of mature CD3⁺ T cells as well as CD56⁺, CD3⁻ NK cells (Fig. 5 B). These results present strong evidence for a common T/NK cell progenitor, since the T cells in these mFTOC were derived from clones that demonstrated 100% NK cell differentiation capabilities.
Discussion

Recent studies (2, 4) in the mouse have suggested the existence of a bipotential T/NK cell progenitor in the thymus, capable of generating T and NK cells in vivo. These studies, however, were unable to determine if there exists a common T/NK progenitor due to the inability to analyze the progenitor populations at the clonal level from freshly isolated cells. The data in the present report clearly demonstrate that in the human fetal thymus the CD34<sup>bright</sup> TN thymocytes possess high NK cell clonogenic frequencies and NK and T cell reconstitution capabilities in mFTOC. Likewise, CD34<sup>bright</sup> TN thymocyte derived clones, before commitment to the NK cell lineage, can differentiate into T cells in mFTOC. These observations support the hypothesis that CD34<sup>bright</sup> TN thymocytes are bipotential progenitors for T and NK cells. In contrast, the CD34<sup>dim</sup> TN thymocytes, the majority of which coexpress CD1, are T cell-committed progenitors incapable of differentiation towards the NK cell lineage. Therefore the expression of CD1 on thymocytes may serve as an important cell surface marker indicating irreversible T cell commitment (5). By contrast, the expression of CD5, although a molecule acquired during thymic differentiation, clearly does not indicate irreversible T cell lineage commitment.

Our results suggest that the earliest T/NK committed progenitor in the fetal thymus expresses high surface density CD34

![Figure 4](image-url).

**Figure 4.** NK clones derived from CD34<sup>+</sup> TN thymocyte subsets. CD34<sup>dim</sup>, CD5<sup>+</sup>; CD34<sup>bright</sup>, CD5<sup>+</sup>; and CD34<sup>bright</sup>, CD5<sup>-</sup> TN thymocytes were single cell cloned into Terasaki plates and cultured for 3 mo (A). No clones were obtained from CD34<sup>dim</sup>, CD5<sup>+</sup> TN thymocytes (B). All clones derived from CD34<sup>bright</sup>, CD5<sup>+</sup>; and CD34<sup>bright</sup>, CD5<sup>-</sup> TN thymocytes were NK cells expressing high surface density CD56 without surface CD3 expression (C and E). Cytolytic function of NK clones against the K562 (open bars) and Jurkat (closed bars) targets (D and F). Data from three representative clones are shown at an E/T ratio of 3:1.

![Figure 5](image-url).

**Figure 5.** Generation of NK and T cells from CD34<sup>+</sup> TN thymocyte clones. CD34<sup>bright</sup> TN thymocytes were single cell cloned and cultured in IL-7, SCF, and the human melanoma cell line, MM170. After 5 d in culture, ~25 cells from each of the most rapidly growing clones were pooled and used to reconstitute a single fetal murine thymic organ. The remaining cells from each clone were grown in IL-2-supplemented cloning media

for 4 wk and phenotyped for the expression of CD56 and CD3 surface antigens (A). The reconstituted mFTOC was cultured for 10 d, homogenized, cultured an additional 7 d as previously described, and phenotyped for CD56 and CD3 surface antigens (B). (A) Representative NK cell antigenic phenotype from 1 of 60 clones used to reconstitute a mFTOC. All clones displayed an identical NK cell antigenic phenotype with high surface density CD56 and no detectable surface CD3. (B) Antigenic phenotype of cultured mFTOC cells demonstrating both NK cells (CD56<sup>+</sup>, CD3<sup>-</sup>; 88%) and T cells (CD56<sup>-</sup>, CD3<sup>+</sup>; 12%). Similar results were obtained from two additional lobes. The data are displayed as two color contour maps divided into quadrants based on >95% of the cells residing in the lower left quadrant after staining with isotype control mAbs.
antigen and cytoCD3ε. It is interesting to note that we have identified a small subset of fetal liver cells displaying these characteristics (Fig. 2B). These CD34+, cytoCD3ε+ cells may represent a population of prethymic T/NK progenitors. Recent reconstitution studies employing SCID mice and human thymic organ cultures have demonstrated that fetal liver contains transplantable T cell progenitors (13, 26). Further studies of the fetal liver CD34+, cytoCD3ε+ population are required to determine the T/NK progenitor capabilities of these cells.

Previous studies (27) have suggested that the human thymus may contain hematopoietic stem cells, capable of multilineage differentiation. Most CD34Brth TN thymocytes are clearly not multipotential stem cells, particularly since the majority of these cells express high levels of cytoCD3ε protein, an unequivocal marker for T cell and/or NK cell lineage commitment (3, 7, 24). Consistent with these findings, it was recently shown that CD34+, CD7+ TN thymocytes are incapable of monocytic/myeloid differentiation (28). It is possible, however, that a small percentage of CD34Brth TN thymocytes (<2% of CD34 expressing cells) that do not express cytoCD3ε protein may represent uncommitted multipotential progenitors. Indeed, in preliminary experiments we have observed that, under appropriate in vitro clonal conditions (12, 13), CD34Brth TN thymocytes can generate myeloid CFUs and dendritic-like cells with a very low cloning frequency <2% (data not shown).

The identification of a bipotential T/NK cell progenitor in the fetal thymus establishes a direct lineage relationship between the T and NK cell differentiation pathways. Based on our observations and others, we propose that CD34Brth, cytoCD3ε expressing TN thymocytes are a relatively homogeneous population of bipotential T/NK cell progenitors. Under the influence of the thymic microenvironment, these progenitors gradually acquire surface CD2, CD5, CD28, CD38, and eventually CD1 antigens (Fig. 6). As these antigens increase in surface density, CD34 is concurrently downregulated and eventually CD1 antigens are detectable in the embryonic liver as early as 6 wk gestation, which is substantially earlier than the formation of the thymus (7). It is possible, therefore, that before the development of the thymic microenvironment, putative T/NK progenitors in fetal liver are directed towards the NK differentiation pathway. The signals which direct these bipotential progenitors towards T and/or NK cell differentiation pathways are at present unknown. The identification of a common T/NK cell progenitor in the thymus, however, will greatly facilitate our understanding of the biochemical and molecular mechanisms that direct the divergent lineage differentiation of NK and T cells.

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