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 CD3ε (cytoCD3ε) protein, can be divided into high ($CD34^{Bright}$) and low ($CD34^{Dim}$) surface expressing populations. $CD34^{Bright}$ 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, $CD34^{Bright}$ 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. $CD34^{Dim}$ 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

[1] *Abbreviations used in this paper:* cytoCD3ε, cytoplasmic CD3ε; mFTOC, murine fetal thymic organ culture; SCF, stem cell factor; TN, triple negative.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/08/0569/08 $2.00
Volume 180 August 1994 569-576

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 $CD4^+$, $CD8^+$, and glycophorin A^+ cells by magnetic bead treatment. Three-color cell 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-CD34 mAbs, fixed, and then incubated with FITC-labeled anti-CD3ε mAb, as previously described (3). Leu 4 mAb was used to detect cytoplasmic CD3 proteins (7). The Leu 4 mAb recognizes CD3ε 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 c-*kit* 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. Zurawski). Clones were established using Yssel's media (11) supplemented with 10% FCS (JR Scientific, Woodland, CA) and 2% human AB serum (Pel-Freez, Brown Deer, WI) supplemented with the indicated factors.

In some experiments, clones were initially established in IL-7, stem cell factor (SCF), and 200 cells per well of an irradiated (5,000 rad) melanoma cell line, MM170 (kindly provided by Dr. Hilary Warren, Woden Valley Hospital, Canberra, Australia) without IL-2. These particular culture conditions were shown to give maximal clonal proliferation of $CD34^{Bright}$ TN thymocytes in short term culture (5 d) without NK cell differentiation. After 5 d in culture, ~25 cells from each of the most rapidly growing 40–60 clones were pooled and used to reconstitute a single murine thymic organ. The remaining cells of each clone were then fed with IL-2–containing media and culture for 4 wk. In some experiments, irradiated allogenic PBMC, JY (B-LCL), IL-2 (100 U/ml), and PHA (0.1 ng/ml) (IL-2 stimulation conditions) were used as previously indicated (6, 11). Growth of dendritic and myeloid cells from progenitor thymic populations was performed according to previously published protocols (12, 13).

Cell-mediated Cytotoxicity. Cytotoxic activity was measured in a standard 4-h ^{51}Cr radioisotope release assay (14) using NK-sensitive K562 (erythroleukemia) and Jurkat (T cell leukemia) cells as targets.

Thymic Organ Culture. 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 $CD34^{Bright}$ 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 ($CD34^{Dim}$), while coexpressing high surface density CD5, CD2, CD7, CD28, and CD38 antigens (Fig. 2 *A*). These $CD34^{Dim}$ thymocytes also expressed CD1, an antigen associated with thymic T cell differentiation (5, 16). The antigenic phenotype of $CD34^{Dim}$ 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 ($CD34^{Bright}$). Unlike the $CD34^{Dim}$ thymocytes, the $CD34^{Bright}$ subset displayed a more immature antigenic phenotype with variable expression of CD5, CD2, CD28,

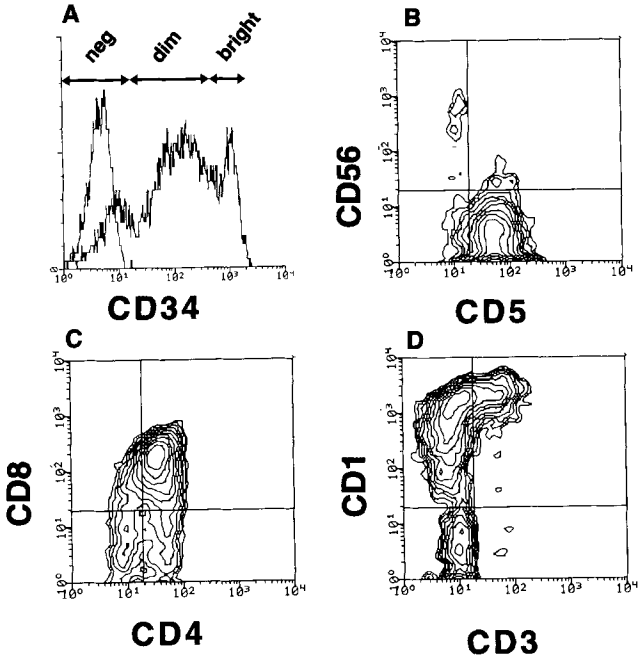

Figure 1. Generation of T and NK cells from CD34$^+$ TN fetal thymocytes in mFTOC. (*A*) Expression of CD34 on fresh fetal TN thymocytes. TN thymocytes can be divided into CD34^{Negative}, CD34^{Dim}, and CD34^{Bright} subsets. Sorted CD34$^+$ TN thymocytes (including both *CD34^{Dim}* and *CD34^{Bright}* subsets) were transferred into mFTOC, and after 10 d in culture, were phenotyped for the expression of CD56 and CD5 (*B*), CD8 and CD4 (*C*), and CD1 and CD3 (*D*) on gated human CD45$^+$ cells. In this representative experiment, 4% of the cells were identified as CD56$^+$, CD5$^-$ NK cells. The histogram of cells stained with control mAb is superimposed over the histogram of cells stained with anti-CD34 mAb (*A*). (*B–D*) 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.

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. 2 *A*). 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 CD3ε (cytoCD3ε) protein. CytoCD3ε expression is unequivocally restricted to cells committed to the T and NK cell lineages, and has never been reported in the B cell or myeloid differentiation pathways (7, 24). Fig. 2 *B* clearly demonstrates that the majority of CD34 expressing TN thymocytes contain high levels of cytoCD3ε, as detected with the Leu-4 mAb, indicating their commitment to the T and/or NK cell lineages. Previous studies (3, 10) have shown that the Leu-4 anti-CD3 antibody recognizes CD3ε protein only when complexed with CD3γ and/or CD3δ proteins. The CD34$^+$ TN thymocytes thus express multiple components of the CD3 complex. In contrast, the majority of CD34$^+$ fetal liver cells did not contain cytoCDε (Fig. 2 *B*). These observations are consistent with the fetal liver being the primary site of fetal erythroid and myeloid differentiation. It is interesting to note that a small percentage (<0.5%) of fetal liver CD34$^+$ cells expressed cytoCD3ε (Fig. 2 *B*). These CD34$^+$ fetal liver cells expressing cytoCD3ε may represent prethymic T and/or NK cell committed progenitors.

Identification of a T/NK Thymic Progenitor Population. Although several antigens were expressed differentially on CD34^{Dim} and CD34^{Bright} TN thymocytes (Fig. 2), CD5 is an antigen expressed on all stages of T cell differentiation and has never been reported on mature NK cells or NK cell precursors (1, 6, 7). We examined whether the expression of CD5 may discriminate CD34$^+$ TN progenitors committed on the T cell lineage. 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. 3 *A*). 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 (CD56$^+$, CD5$^-$, surface CD3$^-$). Lobes reconstituted with CD34^{Dim}, CD5$^+$ 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, *B* and *C*). In contrast, the CD34^{Bright} TN thymocyte subset, regardless of the expression of CD5, was capable of generating both T and NK cells in mFTOC (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.

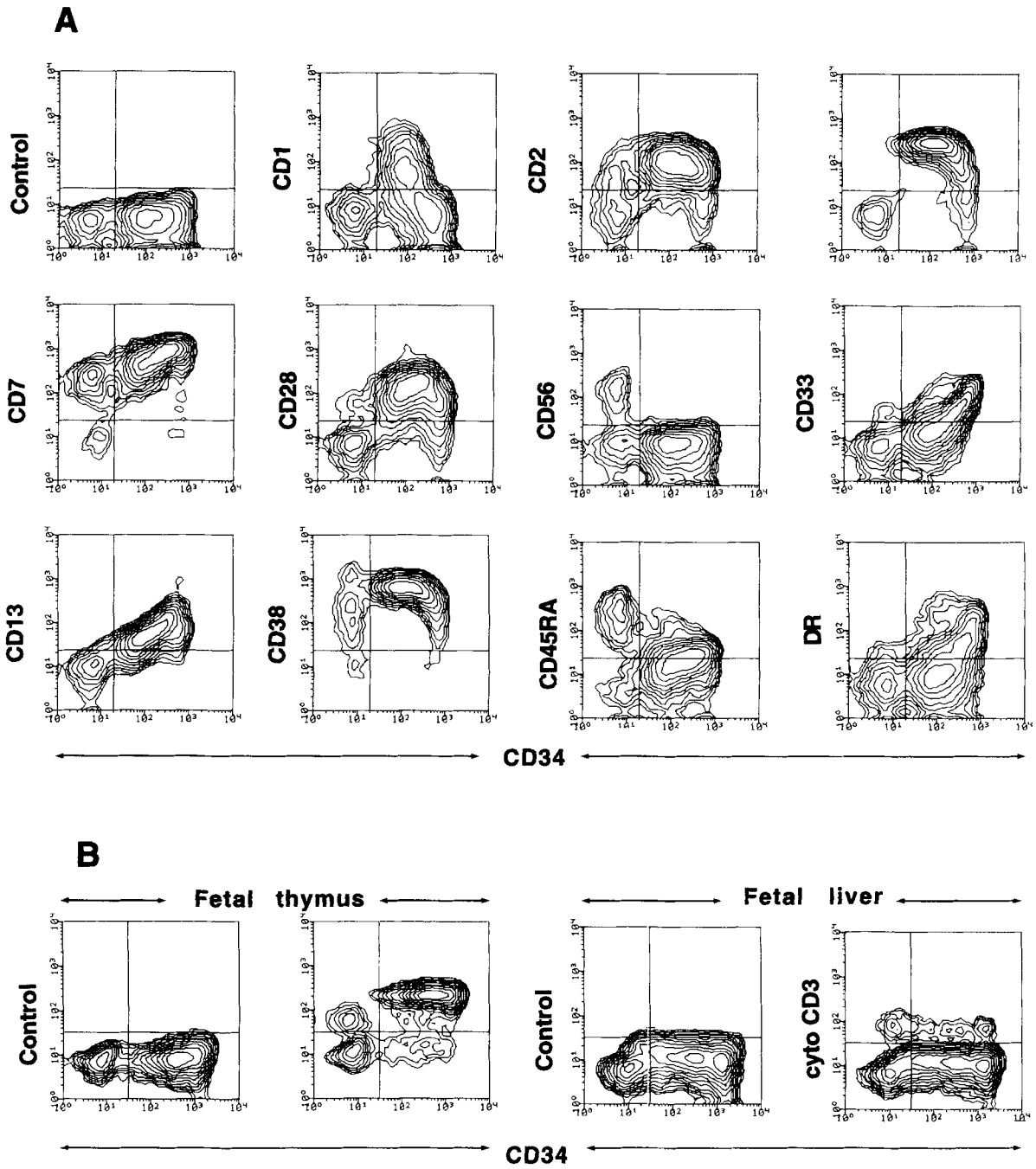

Figure 2. Antigenic phenotype of fetal CD34$^+$ TN thymocytes. (*A*) Freshly isolated fetal TN thymocytes were stained with Cy-chrome–conjugated anti-CD45 mAb, FITC-conjugated anti-CD34 mAb, and PE-conjugated mAbs against various leukocyte antigens. (*B*) TN thymocytes and T cell–depleted fetal liver cells were analyzed for the coexpression of surface CD34 and cytoCD3ϵ antigens on CD45$^+$ gated 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 CD34^{Bright}, CD5$^+$, and the CD34^{Bright}, CD5$^-$ subsets had expanded sufficiently to allow for analysis of function, antigenic phenotype and TCR gene rearrangement. All clones derived from the CD34^{Bright} 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 CD34^{Bright} TN thymocytes were combined into 10 pools and analyzed

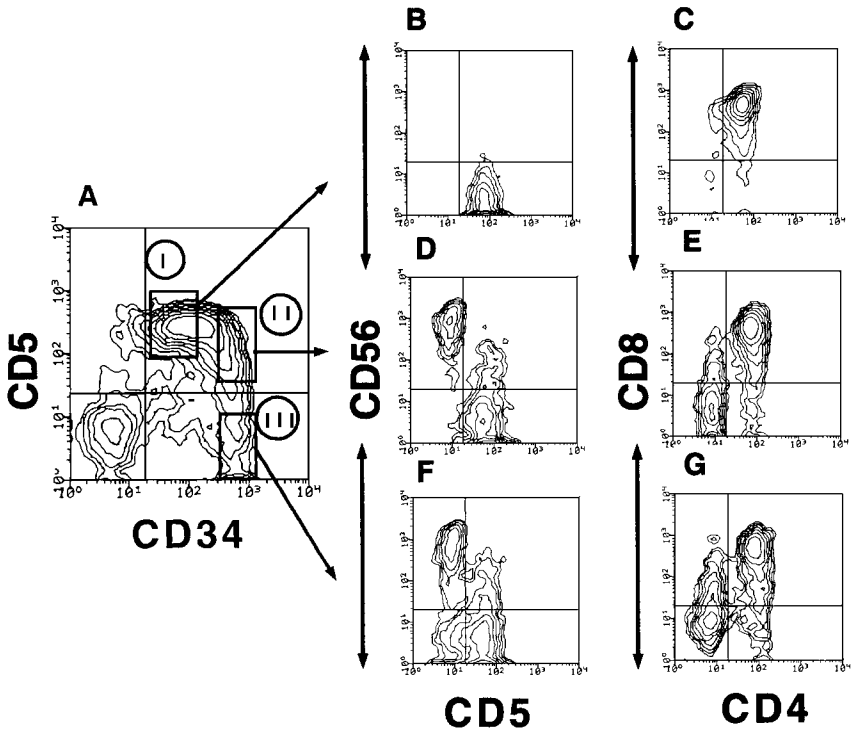

Figure 3. Antigenic phenotype of human thymocytes recovered from mFTOC. TN thymocytes were stained with FITC-conjugated anti-CD34 and PE-conjugated anti-CD5 mAbs and sorted (>97% purity) into three subsets: I-CD34^{Dim}, CD5$^+$; II-CD34^{Bright}, CD5$^+$; and III-CD34^{Bright}, CD5$^-$. (*A*) Each sorted 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 CD56$^+$, CD5$^-$ NK cells. (*D* and *E*) Expression of CD56, CD5, CD4, and CD8 on cells recovered from mFTOC reconstituted with II-CD34^{Bright}, 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-CD34^{Bright}, 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 CD34^{Bright} thymocytes were NK cells. Similar percentages of NK cells were obtained from lobes seeded with CD34^{Bright} cells regardless of expression of CD5.

by Southern blot for TCR gene rearrangements. No evidence of TCR Cβ or Jγ 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 CD34^{Bright} TN Thymocytes Clones. Although CD34^{Bright} 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 CD34^{Bright} TN thymocytes, CD34^{Bright} 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 CD34^{Bright} 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.

Table 1. *Frequency of Clones Obtained from Thymic Populations*

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

TN thymocytes were cloned as indicated in Fig. 4.
* Cultures supplemented with indicated factors (refed 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.

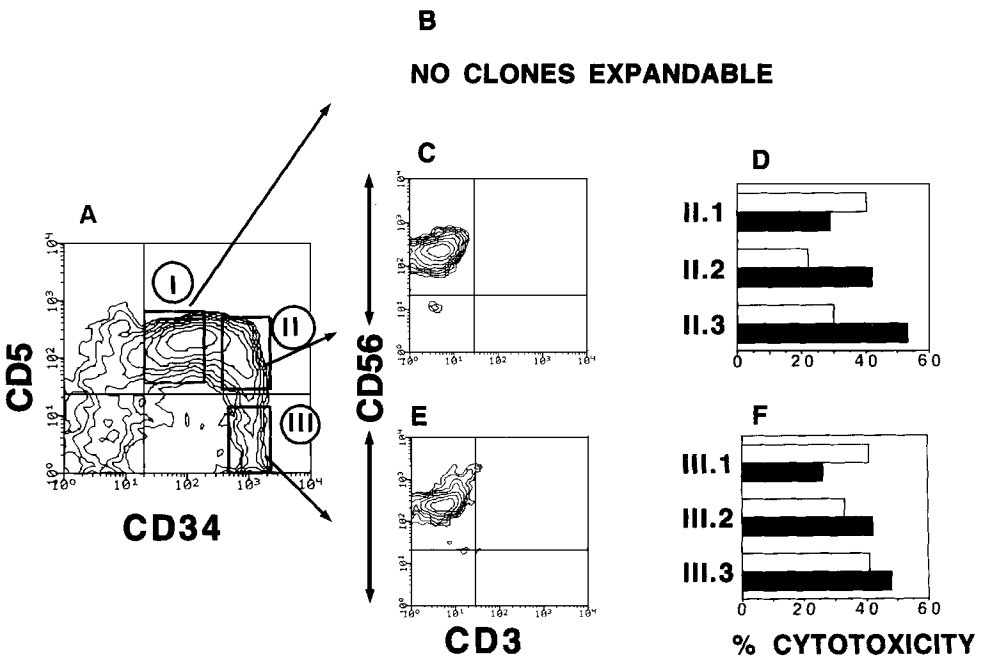

Figure 4. NK clones derived from CD34$^+$ TN thymocyte subsets. CD34^{Dim}, CD5$^+$; CD34^{Bright}, CD5$^+$; and CD34^{Bright}, CD5$^-$ TN thymocytes were single cell cloned into Terasaki plates and cultured for 3 mo (*A*). No clones were obtained from CD34^{Dim}, CD5$^+$ TN thymocytes (*B*). All clones derived from CD34^{Bright}, CD5$^+$; and CD34^{Bright}, CD5$^-$ 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.

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^{Bright} TN thymocytes possess high NK cell clonogenic frequencies and NK and T cell reconstitution capabilities in mFTOC. Likewise, CD34^{Bright} 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^{Bright} TN thymocytes are bipotential progenitors for T and NK cells. In contrast, the CD34^{Dim} 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

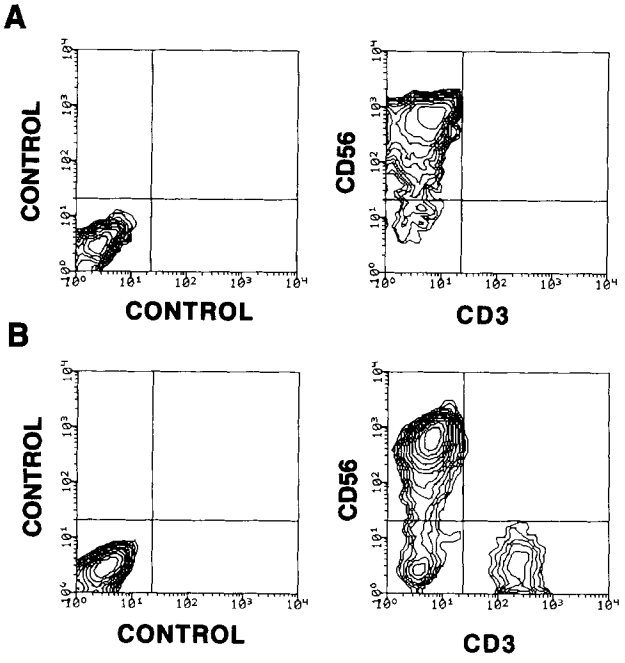

Figure 5. Generation of NK and T cells from CD34$^+$ TN thymocyte clones. CD34^{Bright} TN thymocytes were single cell cloned and cultured in IL-7, SCF, and the human melanoma cell line, MM170. After 5 d in culture, ~25 cells from each of the most rapidly growing clones were pooled and used to reconstitute 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$^+$, CD3$^-$; 88%) and T cells (CD56$^-$, CD3$^+$; 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.

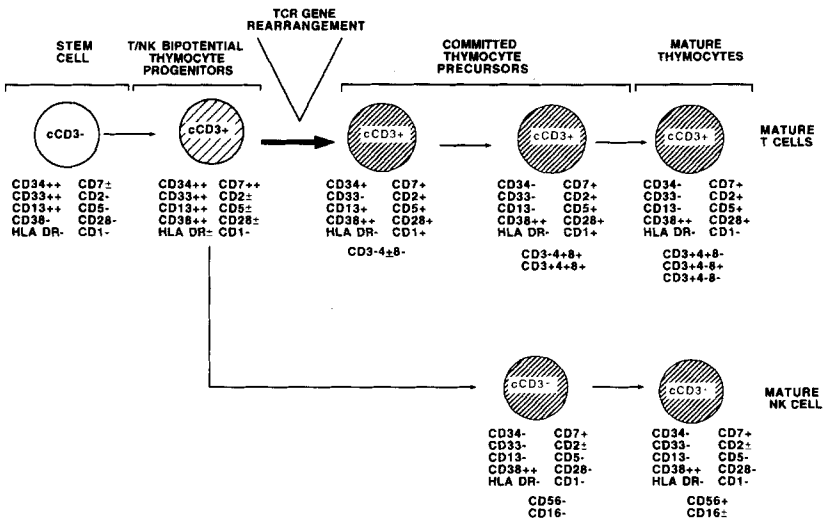

Figure 6. Model of fetal thymocyte development. Hypothetical fetal thymic stem cell (<1% of TN thymocytes) express high levels of CD34, CD13, and CD33 antigens. Commitment of the pluripotent $CD34^{++}$, $CD13^{++}$, and $CD33^{++}$ cells toward the T/NK cell lineage induces the expression of cytoplasmic CD3 proteins (cCD3) and CD7. Under the influence of the thymic environment, CD2, CD5, and CD28 are subsequently expressed on the bypotential T/NK progenitor. Downregulation of CD34, CD13, and CD33 followed by expression of CD1 and TCR gene rearrangement indicates irreversible commitment to the T cell lineage. $CD1^+$ T cell progenitors preferentially expand (*thick arrow*) and further mature in the thymus. Limited intrathymic NK cell differentiation results in the downregulation of CD34, CD13, CD33, CD5, CD28, and cCD3 and the progressive acquisition of NK cell–associated markers. Pluripotent stem cells (*open circle*), bipotential progenitor cells (*low density dashed circle*), lineage committed cells (*high density dashed circles*).

antigen and cytoCD3ϵ. It is interesting to note that we have identified a small subset of fetal liver cells displaying these characteristics (Fig. 2 *B*). These $CD34^+$, $cytoCD3\epsilon^+$ 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 $CD34^{Bright}$ 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 $CD34^{Bright}$ 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), $CD34^{Bright}$ 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 $CD34^{Bright}$, cytoCD3ϵ expressing TN thymocytes are a relatively homogenous 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 these progenitors become irreversibly committed to the T cell lineage, presumably at the onset of TCR rearrangement. We predict that the initiation of TCR rearrangement occurs during the transition from $CD1^-$ to $CD1^+$ in TN thymocytes. Before the expression of CD1, $CD34^{Bright}$ TN thymocytes have the potential to differentiate towards either the T or NK cell lineages. Although the majority of thymic progenitors are destined for commitment to the T cell lineage, a small percentage of these bipotential progenitors differentiate into committed NK cell precursors and mature NK cells within the microenvironment of the thymus. The immunological significance of thymic NK cell differentiation is at present unknown. Mature NK cells, however, are detectable in the embryonic liver as early as 6 wk gestation, which is substantially earlier than the formation of the thymus anlage (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 the biochemical and molecular mechanisms that direct the divergent lineage differentiation of NK and T cells.

We thank James Cupp, Josephine Polakoff, and Eleni Callas for expert assistance with the flow cytometry; and Jackie Kennedy and Gary Laramee for their help with Southern blotting.

DNAX Research Institute is supported by Schering Plough Corporation.

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Received for publication 25 January 1994 and in revised form 25 April 1994.

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