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Human Natural Killer Cell Committed Thymocytes and Their Relation to the T Cell Lineage

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Summary

Recent studies have demonstrated that mature natural killer (NK) cells can be grown from human triple negative (TN; CD3⁻, CD4⁻, CD8⁻) thymocytes, suggesting that a common NK/T cell precursor exists within the thymus that can give rise to both NK cells and T cells under appropriate conditions. In the present study, we have investigated human fetal and postnatal thymus to determine whether NK cells and their precursors exist within this tissue and whether NK cells can be distinguished from T cell progenitors. Based on the surface expression of CD56 (an NK cell-associated antigen) and CD5 (a T cell-associated antigen), three phenotypically distinctive populations of TN thymocytes were identified. CD56⁺, CD5⁻; CD56⁻, CD5⁻, and CD56⁻, CD5⁺. The CD56⁺, CD5⁻ population of TN thymocytes, although displaying a low cytolytic function against NK sensitive tumor cell targets, were similar in antigenic phenotype to fetal liver NK cells, gave rise to NK cell clones, and were unable to generate T cells in mouse fetal thymic organ cultures (mFTOC). This population of thymocytes represents a relatively mature population of lineage-committed NK cells. The CD56⁻, CD5⁻ population of TN thymocytes were similar to thymic NK cells in antigenic phenotype and NK cell clonogenic potential. Clones derived from this population of TN thymocytes acquired CD56 surface expression and NK cell cytolytic function. CD56⁻, CD5⁻ TN thymocytes thus contain a novel population of NK cell-committed precursors. The CD56⁻, CD5⁻ population of TN thymocytes also contains a small percentage of CD34⁺ cells, which demonstrate no *in vitro* clonogenic potential, but possess T cell reconstituting capabilities in mFTOC. The majority of TN thymocytes do not express CD56, but coexpress CD34 and CD5. These CD56⁻, CD5⁺, CD34⁺ cells demonstrate no NK or T cell clonogenic potential, but are extremely efficient in repopulating mFTOC and differentiating into CD3⁺, CD4⁺, CD8⁺ T cells. The results of this investigation have identified NK cells and NK cell precursors in the human thymus and have shown that these cell types are unable to differentiate along the T cell lineage pathway. Thus, while a common NK/T cell progenitor likely exists, once committed to the NK cell lineage these cells no longer have the capacity to develop along the T cell developmental pathway.

Human T cell development involves a series of intrathymic events during which fetal liver and/or bone marrow-derived progenitor cell populations migrate to the thymic anlage, and undergo a poorly understood process of differentiation (1-4). It is generally accepted that the earliest T cell precursors in the thymus lack the expression of CD3, CD4, and CD8 and are thus referred to as triple negative (TN)¹ thymocytes (5-10). It has recently been demonstrated that the majority of these human TN thymocytes express the progenitor cell-associated antigen CD34 (11) and are capable of

developing into mature T cells in human thymic organ cultures (12). CD34⁺ fetal liver and bone marrow progenitors have also been shown to reconstitute human fetal thymic implants in the SCID/Hu mice (13).

NK cell differentiation, however, is at present poorly understood, primarily due to the lack of a specific organ for maturation and a defined antigen receptor on these cells. The differentiation and development of NK cells and their relationship to the T cell lineage remains controversial. Although it has been described that NK cells are present in both the human (14) and the mouse thymus (15), it is generally believed that the development of NK cells is independent of the thymic microenvironment, particularly since NK cells develop normally in athymic mice (16), and differentiated NK

¹ Abbreviations used in this paper: mFTOC, mouse fetal thymic organ culture; TN, triple negative.

cells are present during human fetal development before the appearance of the thymic anlage (17). Nevertheless, different subsets of human T and NK cells share lytic activity (18) and express many membrane antigens in common, including CD2, CD7, CD8, CD16, and CD56 (19). It has also been reported that NK cells can express cytoplasmic CD3 proteins (19, 20) and nonfunctional transcripts for the TCR chains (21). Despite these similarities, NK cells do not express the T cell-associated antigen CD5 or rearrange the TCR genes (17, 22).

Due to these phenotypic and functional similarities, it has been proposed that NK cells are related to the T cell lineage and may indeed share a common T/NK cell-committed progenitor. This hypothesis has been supported by the ability of human total thymocytes (23–26) and TN thymocytes to generate NK and T cells under appropriate in vitro conditions (27, 28) and by the ability of murine Fc γ RII/III⁺ TN thymocytes to generate NK cells and T cells in vivo (29). The identification of a common T/NK cell progenitor remains elusive, since previous studies have employed heterogeneous populations of thymocytes that may contain several distinctive lineage-committed progenitor populations.

In the present study, we have attempted to clarify the relationship between NK cells and T cells in the thymus by phenotypically dissecting human TN thymocytes and determining the capacity of various subpopulations to generate NK cells in vitro and T cells in thymic organ cultures. Our results show that CD56⁺, CD5⁻, CD34⁻ and CD56⁻, CD5⁻, CD34⁻ TN thymocytes include NK and NK cell-committed precursors, respectively, incapable of generating T cells in mouse fetal thymic organ cultures (mFTOC). CD34⁺ TN thymocytes, on the other hand, are incapable of generating NK cells using in vitro cloning conditions, while efficiently differentiating to T cells in mFTOC.

Materials and Methods

Cell Preparations. Fetal thymic and liver tissue were obtained from elective therapeutic abortions. Gestation age was determined by crown-rump length and ranged from 13 to 24 wk. Newborn thymic tissue was obtained from normal pediatric subjects undergoing median sternotomy and corrective cardiovascular surgery. 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 (17). Thymocyte cell suspensions were prepared, and separation of different thymic populations was performed by a combination of magnetic bead depletion and cell sorting, as previously described (11). Newborn and fetal thymic tissue were used interchangeably in this study, since similar results were obtained in all experiments regardless of the maturational stage of the thymic tissue.

mAb mAbs against the following antigens were used: CD1a (T6; Coulter Corp., Hialeah, FL); CD2 (Leu 5b); CD3 (Leu 4); CD4 (Leu3a+b, L77); CD5 (Leu 1, UCHT2; PharMingen, San Diego, CA); CD7 (Leu 9); CD8 (Leu 2a); CD10 (Calla); CD11a (L7); CD11b (Leu 15); CD11c (Leu-M5); CD13 (Leu-M7); CD14 (Leu-M3); CD16 (Leu 11a); CD18 (L130); CD19 (Leu 12); CD20 (Leu-16); CD28 (L293); CD33 (Leu-M9); CD34 (HPCA-2); CD38 (Leu 17); CD45 (HLE, H130, PharMingen); CD45RA (Leu 18); CD56 (Leu 19 and L185); glycophorin A (10F7Mn; American Tissue

Culture Center, Rockville, MD); and mouse CD45 (30F11.1, PharMingen). mAbs were generously provided by Becton Dickinson Immunocytometry System (San Jose, CA), unless otherwise indicated. Fluorochrome conjugated mouse Ig isotype-specific controls were from Becton Dickinson Immunocytometry System and PharMingen. Cy-Chrome™ conjugated streptavidin was purchased from PharMingen.

Immunofluorescence, Flow Cytometry, and Cell Sorting. Methods of immunofluorescence staining and cell sorting have been described previously (17, 30). Enrichment of selected thymic populations was performed by incubation with specific mAb followed by magnetic bead depletion (DynaL Inc., Oslo, Norway) as previously described (11). This procedure was repeated two or three times until the purity of the cells, monitored by staining with FITC goat anti-mouse Ig (Caltag Labs., So. San Francisco, CA) was >90%. Three-color immunofluorescence was performed with PE, FITC, and Cy-Chrome™ fluorochrome-conjugated mAbs, followed by flow cytometric analysis. A gate was set on each subpopulation according to the expression of CD56 and CD5 and the percentage of cells bearing the third differentiation Ag was determined. Appropriate fluorochrome-conjugated isotype-matched control Igs were used in all experiments. Samples were analyzed using a FACScan® (Becton Dickinson Immunocytometry System). In some experiments, bead depletion was performed with FITC-conjugated mAbs to ensure the purity of the cell subpopulations. Thus, any cell not removed by magnetic bead depletion would be subsequently detected and excluded by flow cytometry.

For cell sorting, thymocytes were depleted of CD4⁺, CD8⁺, and glycoprotein A⁺ cells by two rounds of magnetic bead treatment. Three-color cell sorting was performed by excluding FITC positive cells. FITC-conjugated anti-CD3, -CD4, -CD8, -CD14, and -CD19 mAbs were used in cell sorting experiments to prepare TN thymocytes.

Cloning of TN Thymic Populations. After magnetic bead depletion of CD4⁺ and CD8⁺ cells, thymocytes were stained with FITC-conjugated anti-CD3, -CD4, -CD8, -CD14, and -CD19, PE-conjugated anti-CD56 mAb and Cy-Chrome™-conjugated anti-CD5 mAb. Thymocytes from each population were cloned at one cell per well in 96-well plates (Costar Corp., Cambridge, MA) using a single cell deposition system of the FACStar Plus® flow cytometer. Clones were established using culture conditions previously described (31) with minor modifications. Briefly, cells were seeded in culture plates containing 5 × 10⁴ irradiated peripheral blood mononuclear cells (PBMC) and 5 × 10³ irradiated JY (EBV transformed B cells) as feeder cells and 0.1 μg/ml PHA (Burroughs Wellcome Co., Research Triangle, NC) in 200 μl of Yssel's media supplemented with 10% FCS (JR Scientific, Woodland, CA) and 2% human AB serum (Pel-Freez, Brown Deer, WI). 7 d later IL-2 (DNAX Research Institute) was added (100 U/ml). Clones were analyzed for the expression of CD56, CD3, and CD16 after 2–3 wk of culture. The antigenic phenotype and lytic activity was determined weekly. Clones were fed every 3 d with IL-2 and restimulated every week with irradiated feeder cells.

Cell-mediated Cytotoxicity. Cytotoxic activity was measured in a standard 4-h ⁵¹Cr radioisotope release assay (32) using NK-sensitive K562 erythroleukemia cells as targets.

Thymic Organ Culture. For the in vitro T cell development of human TN thymocytes we used the mouse fetal thymic organ culture system previously described by Fisher et al. (33), with minor modifications. Briefly, thymuses were removed from embryos of BALB/c mice on day 15 of gestation and treated with 1.35 mM 2'-deoxyguanosine (dGu) (Sigma Chemical Co., St. Louis, MO) for 5 d. Human CD45⁺ thymic TN populations were sorted to

>95% purity. The thymic lobes were cocultured with sorted human thymic TN population in Iscove's Modified Dulbecco Media (IMDM). (GIBCO Laboratories, Grand Island, NY), containing 2% human AB serum, as previously described (33, 34). The mice lobes with the human cells were placed on nucleopore filters (0.8 μ m, Costar Corp.) over gelfoam rafts (Upjohn Company, Kalamazoo, MI) and cultured for 15 d. At the end of the incubation periods, thymic lobes were homogenized in PBS and analyzed. Three-color immunofluorescence was performed by incubating the cells with Cy-Chrome™-conjugated anti-human CD45 mAb, PE anti-CD8, FITC anti-CD3, and FITC anti-CD4. In some experiments anti-mouse CD45 mAb was used to detect residual mouse leukocytes.

Results

NK Cells Are Present in the Human Thymus and Do Not Differentiate to T Cells. Thymocytes from fetal (13–23 wk of gestation age) and neonatal thymuses were examined for the expression of the NK cell-associated antigen CD56 and the T cell-associated antigen CD5. CD5 is expressed during all differentiation stages of human T cell development, including thymocytes and circulating T cells (11, 35) and has never been observed on fetal or adult NK cells (17). Thus, analyzing the expression of CD56 and CD5 on TN thymocytes allows for the dissociation of NK cells (CD56⁺, CD5⁻) from T cells (CD56⁻, CD5⁺). A minor population of CD56⁺, CD5⁻ cells (<0.05%) was detected in neonatal thymus and fetal thymus after 20 wk of gestation (Fig. 1 B). At earlier developmental stages (13–20 wk of gestation), the CD56⁺, CD5⁻ population comprised a greater percentage (0.4–0.1%) of CD45⁺ thymocytes. The CD56⁺, CD5⁻ thymocytes were primarily contained within the CD3⁻, CD4⁻, CD8⁻ population. Therefore, to enrich for CD56⁺, CD5⁻ cells, thymocytes were depleted of T cells (CD3⁺, 4⁺, 8⁺), B cells (CD19⁺), and monocytes (CD14⁺). The resulting cell population, which will subsequently be referred to as TN thymocytes, comprised 5–10% of total thymocytes, and contained 3–15% CD56⁺, CD5⁻ cells (Fig. 1 C).

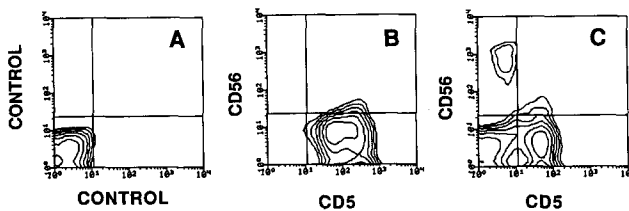


Figure 1. CD56 and CD5 expression on total and TN thymocytes. Human fetal thymocytes and TN thymocytes from an 18-wk gestation fetus were prepared as described in Materials and Methods and analyzed by three-color flow cytometry for the expression of CD56, CD5, and CD45 antigens. Two-color contour maps of CD45⁺-gated cells were divided into quadrants based on >95% of the cells residing in the lower left quadrant after staining with isotype control mAbs (A). Of total CD45⁺ thymocytes, CD56⁺, CD5⁻ cells comprised <0.05% of the total thymocytes (B). Three cell populations were observed in TN thymocytes preparations (C); CD56⁺, CD5⁻ (range 3–15%); CD56⁻, CD5⁻ (range 1–10%); and CD56⁻, CD5⁺ (range 75–96%).

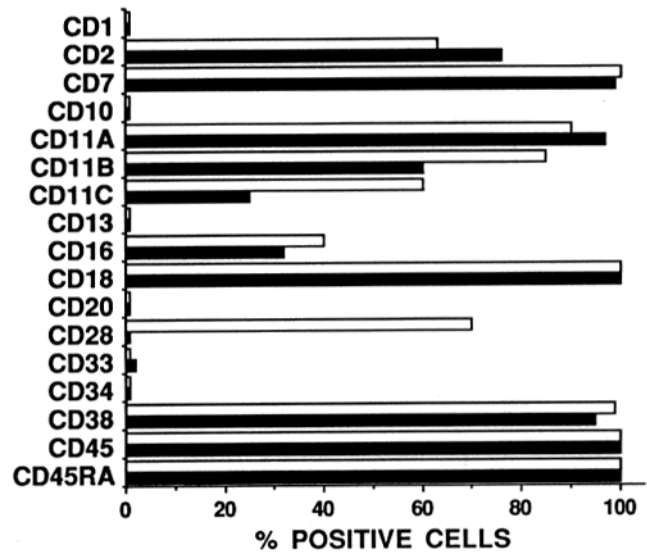


Figure 2. Comparative phenotype of fetal liver NK cells and CD56⁺, CD5⁻ TN thymocytes. Fetal liver mononuclear cells (open bars) and TN thymocytes (black bars) were isolated from 15–20-wk-gestation-age fetuses as described in Materials and Methods and were stained by three-color immunofluorescence using mAbs against CD56, CD5, and a panel of leukocyte antigens. By setting a gate on CD56⁺, CD5⁻ cells, it was possible to determine the proportion of cells within each subset that expressed the third differentiation Ag. The percentages shown are averages based on analysis of at least three tissue samples.

A comparison of CD56⁺, CD5⁻ TN thymocytes to fetal liver NK cells revealed remarkable similarities. Analysis of cell surface antigen expression showed that both CD56⁺, CD5⁻ TN thymocytes and fetal liver NK cells expressed CD7, CD11a, CD18, and high surface density CD38, CD45, and CD45RA antigens (Fig. 2). CD2, CD11b, CD11c, and CD16 antigens were expressed on variable percentages of both CD56⁺, CD5⁻ TN thymocytes and fetal liver NK cells. In addition, thymic CD56⁺, CD5⁻ TN cells and fetal liver NK cells did not express the thymic T cell-associated antigen CD1a; the B cell-associated antigen CD20; the monocytic/myeloid-associated antigens, CD13, and CD33; or the progenitor cell-associated antigens CD34 and CD10. Unlike fetal liver NK cells, however, the majority of CD56⁺, CD5⁻ TN thymocytes did not express the T cell-associated antigen, CD28 (Fig. 2).

To determine the cytolytic capabilities of fetal thymic CD56⁺, CD5⁻ TN cells these cells were sorted to greater than 98% purity and assayed for cytolytic activity against the NK sensitive tumor cell target K562 (Fig. 3). CD56⁺, CD5⁻ TN thymocytes demonstrated measurable levels of NK cell-mediated cytotoxicity, albeit lower than that mediated by freshly isolated fetal liver NK cells.

CD56⁺, CD5⁻ TN thymocytes were cloned from fetal and neonatal thymus using cellular cloning techniques that have been previously shown to be optimal for expansion of NK and T cells (17, 31). The cloning frequency of CD56⁺, CD5⁻ TN thymocytes ranged from 1/25 to 1/100 and were

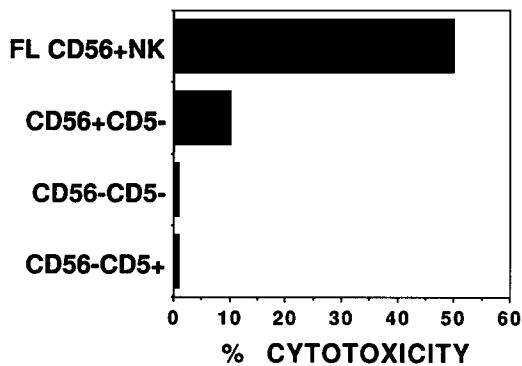


Figure 3. Cytotoxic activity of freshly isolated TN thymic populations. CD56⁺,CD5⁻, CD56⁻,CD5⁻, and CD56⁻,CD5⁺ TN thymocytes were sorted to >95% purity and assayed for cytotoxic activity against the NK cell-sensitive tumor cell target, K562, in a 4-h ⁵¹Cr release assay. Effector to target ratio = 25:1. Representative results from three experiments are presented. Fresh fetal liver NK cells were used as a control for comparison.

comparable to the cloning frequencies observed for fetal liver NK cells. In most experiments, however, neonatal thymic CD56⁺,CD5⁻ TN cells displayed higher cloning frequencies than those observed for fetal CD56⁺,CD5⁻ TN thymocytes. Over 200 fetal and neonatal thymic NK clones were established from more than 10 thymic tissue samples and analyzed for cytolytic function and cell surface antigenic phenotype. All clones established from thymic CD56⁺,CD5⁻ TN thymocytes maintained a typical NK cell antigenic phenotype (CD56⁺,mCD3⁻) (Fig. 4). 40–80% of these NK clones expressed the low affinity IgG Fc-receptor, CD16 (not shown). These clones also demonstrated potent cytolytic activity against the NK-sensitive tumor target, K562 (Fig. 4).

Phenotypic, functional, and cloning experiments clearly indicated that thymic CD56⁺,CD5⁻ TN cells were committed to the NK cell lineage, however, in order to determine if these cells possessed any T cell differentiation capac-

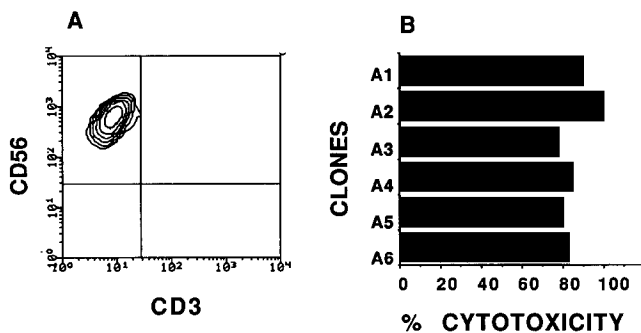


Figure 4. Phenotypic and functional activity of clones derived from CD56⁺,CD5⁻ TN thymocytes. Clones were established as described in Materials and Methods from CD56⁺,CD5⁻ TN thymocytes. The expression of CD56 and surface CD3 (mCD3) was analyzed after 1 mo in culture (A, representative clone). All the clones established from this population were CD56⁺,mCD3⁻. Clones were assayed for cytotoxic activity against K562 in 4-h ⁵¹Cr release assay (E:T = 5:1). The cytolytic function of six representative clones is displayed (B).

ity, CD56⁺,CD5⁻ TN thymocytes were introduced into mFTOC, a system that has previously been shown to support human T cell differentiation and maturation (33). Freshly sorted thymic CD56⁺,CD5⁻ and CD56⁻,CD5⁺ TN thymocytes were introduced into mouse thymic lobes and cocultured for 15 d. After culture, the number of viable cells was determined together with the expression of human CD4, CD8, and CD45 surface antigens. Large numbers of viable cells were recovered from lobes seeded with CD56⁻,CD5⁺, TN thymocytes (16–30-fold cell expansion). Most of the hematopoietic cells isolated from the mouse thymic lobes were of human origin (>90% hCD45⁺). 50% of them were CD3⁺, and 60–90% coexpressed CD4 and CD8 antigens (Fig. 5, A and B). In contrast, thymic CD56⁺,CD5⁻ TN thymocytes failed to reconstitute these organs (Fig. 5, C and D) and the few cells that were recovered were stained with anti-mouse CD45 mAb.

The extremely effective reconstitution capacity of the CD56⁻,CD5⁺ TN thymocyte population to differentiate into CD4⁺,CD8⁺ T cells in mFTOC substantiates the hypothesis that these cells represent the major population of T cell-committed progenitors in the thymus. Previous reports

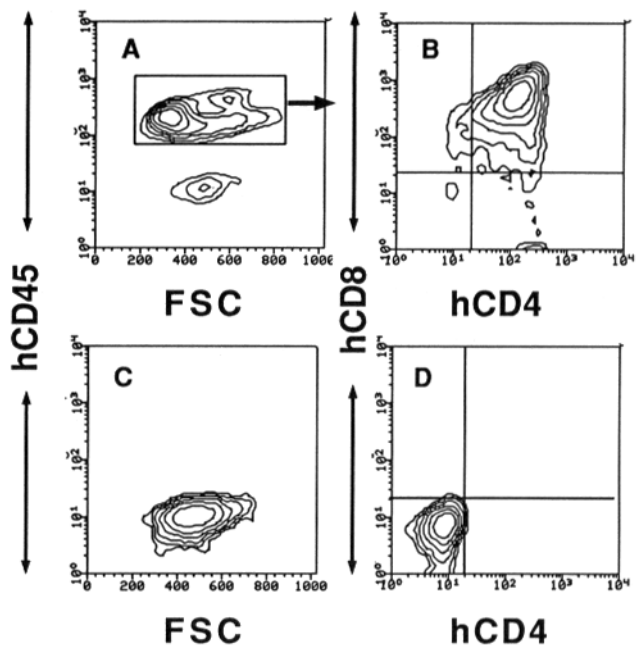


Figure 5. Phenotype of human thymocytes recovered from mFTOC. Three-color sorted CD56⁺,CD5⁻ and CD56⁻,CD5⁺ TN cells were cocultured with mouse fetal thymic lobes. 10³ cells were seeded per lobe. After 15 d in culture the recovered cells (10 lobes) were subjected to three-color immunofluorescence analysis for the expression of human antigens (Cy-Chrome™ conjugated anti-CD45, PE anti-CD8, and FITC anti-CD4). A and B show the surface antigen expression of recovered cells from thymic lobes seeded with CD56⁻,CD5⁺ TN thymocytes. More than 90% are human CD45⁺ (hCD45) (A). 70% of the hCD45⁺ cells coexpressed human CD4 and CD8 (B). C and D show the phenotype of cells recovered from thymic lobes seeded with the CD56⁺,CD5⁻ TN population. Less than 2% were CD45⁺. The hCD45⁻ cells expressed the mouse CD45 antigen, recognized by anti-mouse T200 mAb (not shown).

have shown that CD34⁺ cells from human fetal liver, bone marrow, and thymus contain T cell progenitors capable of reconstituting thymic organ cultures (12, 13). Consistent with these studies, the majority of CD56⁻,CD5⁺ TN thymocytes coexpressed CD34, CD1, and CD28 antigens (Fig. 6) and demonstrated no in vitro clonogenic potential. These results clearly show that the CD56⁺,CD5⁻ and CD56⁻,CD5⁺ TN thymocyte populations are distinct, suggesting that they represent independent NK and T cell-committed thymocyte populations, respectively.

CD56⁻,CD5⁻ TN Thymocytes Include Distinct Precursors for T and NK Cell Lineages. In addition to the CD56⁺,CD5⁻ cells, a significant percentage of TN thymocytes expressed neither CD56 nor CD5 (Fig. 1 C). These CD56⁻,CD5⁻ TN thymocytes were compared by multiparameter flow cytometry to the CD56⁺,CD5⁻ and CD56⁻,CD5⁺ TN populations. As summarized in Fig. 6, CD56⁻,CD5⁻ TN thymocytes expressed CD45, indicating hematopoietic origin, and displayed an antigenic profile very similar to the CD56⁺,CD5⁻ TN thymocytes, except for the expression of CD16. CD16 was not expressed on a significant percentage of CD56⁻,CD5⁻ TN thymocytes (<1%). Similar to CD56⁺,CD5⁻ TN thymocytes, CD1, CD28, and CD34 antigens were not expressed on the majority of CD56⁻,CD5⁻ TN thymocytes (Fig. 6). Interestingly, a very small percentage of CD34⁺ cells (1–10%) was consistently detected within the CD56⁻,CD5⁻ TN population (Fig. 7). Unlike the majority of CD56⁻,CD5⁻ cells, these CD34⁺ cells coexpressed CD28 and low surface density CD45 (data

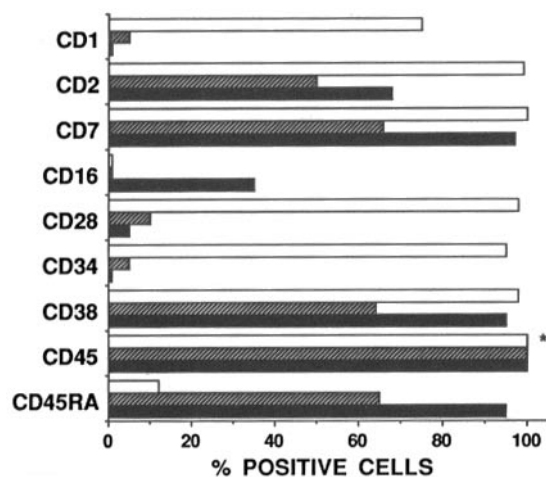


Figure 6. Comparative phenotype of CD56⁺,CD5⁻, CD56⁻,CD5⁻, and CD56⁻,CD5⁺ TN thymocytes. TN thymocytes were prepared as described in Materials and Methods and stained with mAbs against CD56, CD5, and a selected panel of leukocyte antigens. CD56⁺,CD5⁻ (black bars), CD56⁻,CD5⁻ (dashed bars), and CD56⁻,CD5⁺ (open bars) TN populations were analyzed for the various antigens by sequentially setting a gate on each population during analysis. The percentages shown are the results of a single representative experiment. Similar results were obtained from three thymuses. Asterisk indicates that the antigen is expressed at a significantly lower surface density in CD56⁻,CD5⁺ TN cells compared with the other thymic cell subsets (as described in Results).

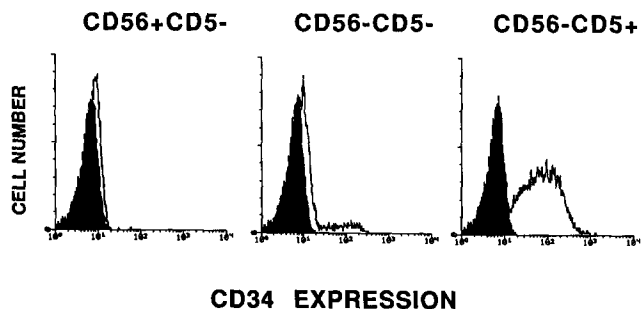


Figure 7. Expression of CD34 on TN thymic populations. After magnetic bead depletion of CD4⁺,CD8⁺ thymocytes, three-color immunofluorescence was performed on CD56⁺,CD5⁻, CD56⁻,CD5⁻, and CD56⁻,CD5⁺ TN population to determine the expression of CD34. CD34⁺ cells comprise <1% of CD56⁺,CD5⁻ TN, 6% of CD56⁻,CD5⁻ TN, and 96% of CD56⁻,CD5⁺ TN cells. Data from a representative experiment are shown. Histograms of CD34 stained cells (open) are superimposed over histograms of isotype Ig control stained cells (black).

not shown). Similar to CD56⁺,CD5⁻ TN thymocytes, freshly sorted CD56⁻,CD5⁻ TN thymocytes demonstrated poor cytolytic activity against the NK-sensitive tumor target, K562 (Fig. 3).

The majority of surface antigens expressed on CD56⁻,CD5⁻ TN thymocytes suggested that this population may contain NK cell-committed precursors. To determine the NK cell differentiation capacity of this thymocyte population, CD56⁻,CD5⁻ TN cells were cloned using culture conditions optimized for the growth of NK and T cells. The cloning efficiencies of both the CD56⁺,CD5⁻ and the CD56⁻,CD5⁻ TN thymocyte subsets were similar, ranging from 1/25 to 1/100. The majority of clones (~70%) established from the CD56⁻,CD5⁻ TN population displayed typical NK cell antigenic phenotype (CD56⁺,mCD3⁻) and showed strong cytolytic activity (Fig. 8, A and B). Interestingly, ~30% of the clones established from the CD56⁻,CD5⁻ TN population expressed variable levels of CD56 (Fig. 8, C and E). The majority of these clones expressed low surface density CD56 (Fig. 8 C) and demonstrated lower cytolytic activity than the typical high surface density expressing clones (Fig. 8 D). Since these clones were established from CD56⁻ cells, the intermediate expression of CD56 on a proportion of cells suggested that these clones may be in the process of in vitro maturation.

A small percentage of the clones established from the CD56⁻,CD5⁻ TN thymocyte population expressed little if any surface CD56 at the time of initial analysis (Fig. 8 E). These particular clones, likewise, showed no apparent cytolytic activity against NK-sensitive tumor targets (Fig. 8 F). Despite the lack of CD56 expression and NK cell cytolytic activity, these clones displayed a typical NK cell phenotype with respect to a large panel of cell surface antigens (data not shown). In order to determine if these clones were capable of NK cell maturation, several CD56⁻ clones were sequentially monitored for the expression of CD56 and the acquisition of NK cytolytic function. As demonstrated in Fig.

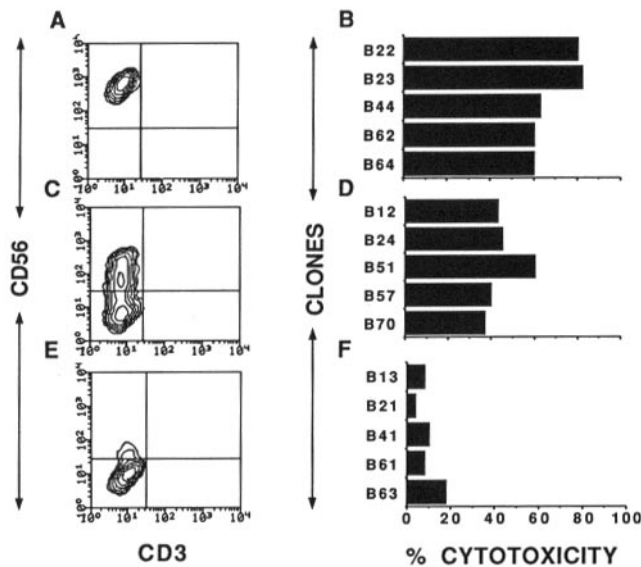


Figure 8. Phenotype and lytic activity of clones derived from CD56⁻, CD5⁻ TN thymocytes. Clones were established as described in Materials and Methods from CD56⁻, CD5⁻ TN thymocytes. Expression of CD56 and mCD3 was analyzed after 1 mo in culture, together with the lytic activity against the NK cell-sensitive tumor cell target, K562. Three general types of clones were observed with varied CD56 expression (A, C, E). The approximate percentage of clones with the indicated phenotype in 15 different cloning experiments was: CD56⁺ bright, 50–100%; CD56⁺ dim, 0–50%; and CD56⁻, 0–5%. Cells stained with isotype Ig controls were negative (>95% in the lower left quadrant, not shown).

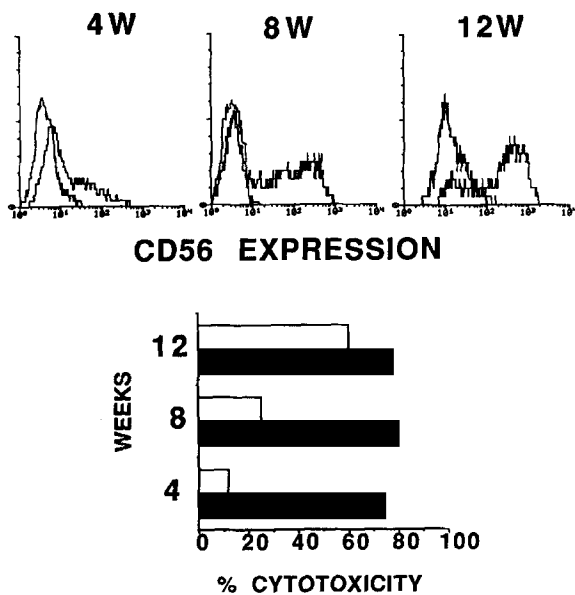


Figure 9. In vitro maturation of CD56⁻ clones. CD56⁻ CD3⁻ clones derived from the CD56⁻ CD5⁻ TN thymocytes were maintained in cloning culture conditions for at least 12 wk. At 4-wk intervals, the expression of CD56 (top histograms) together with the lytic activity against K562 (bottom bar histogram) was monitored. One representative clone is displayed in this figure. Cytotoxicity was assayed against K562 at an E:T = 5:1. The solid bars represent lytic activity of a typical CD56⁺ bright NK cell clone derived from the same thymic population. In the top panel, histogram of anti-CD56 stained cells were superimposed over histograms of Ig control stained cells.

9, many CD56⁻ clones gradually acquired expression of CD56. The acquisition of CD56 was closely correlated with the apparent increase in NK cell cytolytic activity. Very few of the CD56⁻ clones remained CD56⁻ after extensive culturing over several months (4 out of 200). Interestingly, these stable CD56⁻ clones demonstrated extremely high proliferation rates and no detectable cytolytic activity. Whether these CD56⁻ clones are related to the NK cell lineage is at present unknown; however, they may represent stable NK cell precursor clones incapable of in vitro maturation.

Although the CD56⁻, CD5⁻ TN thymocyte population contains NK cell precursors capable of in vitro maturation, CD56⁻ CD5⁻ TN cells may also be capable of T cell differentiation in the thymic microenvironment. Using mFTOC, sorted CD56⁻, CD5⁻, and CD56⁻, CD5⁺ TN thymocyte populations were cocultured with mouse thymic lobes and analyzed 15 d later. Surprisingly, both thymocyte populations were capable of reconstituting thymic organ cultures, although the number of human CD45⁺ cells recovered from each lobe was 5–10-fold greater in lobes seeded with the CD56⁻, CD5⁺ TN thymocyte population (Fig. 10). Since previous studies have shown that CD34⁺ TN

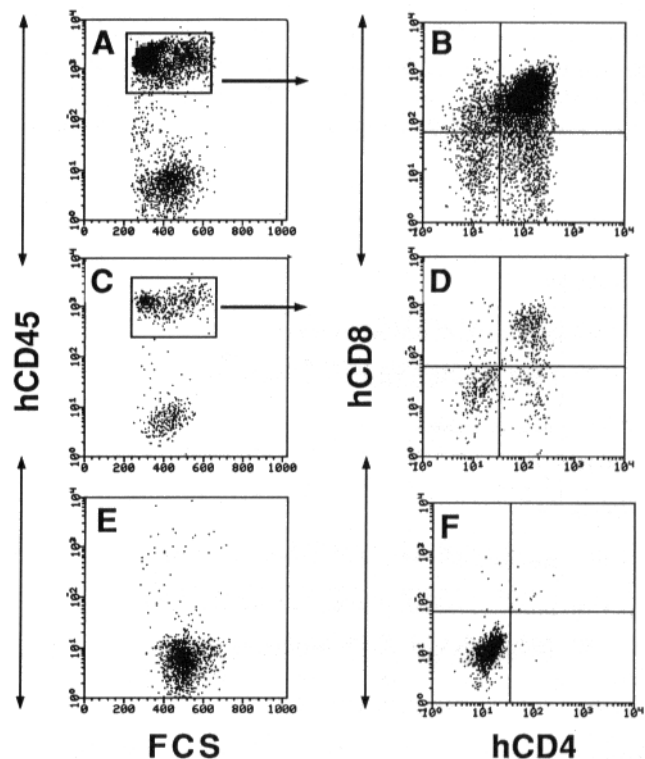


Figure 10. mFTOC reconstitution with CD56⁻, CD5⁻, TN thymocytes. 10³ sorted CD56⁻, CD5⁺ TN cells (A and B), CD56⁻, CD5⁻ TN cells (C and D) and CD56⁻, CD5⁻ TN thymocytes depleted of CD34⁺ cells (E and F), were cocultured with mouse fetal thymic lobes and subsequently stained for the indicated antigens (see Fig. 5, legend). The percentage of human cells was determined by the expression of hCD45 (70% hCD45⁺ cells in A; 74% in C; <2% in E). CD4 and CD8 expression was displayed for the gated hCD45⁺ cells. >95% cells stained with control Ig were in the lower left quadrant of B, D, and F (not shown).

thymocytes are primarily responsible for reconstituting thymic organ cultures (12), we hypothesized that the small percentage of CD34⁺ cells within the CD56⁻, CD5⁻, TN population (Fig. 7) were responsible for the T cell reconstitution capacity of this TN population. To test this hypothesis, reconstitution experiments were performed with sorted CD56⁻, CD5⁻ TN thymocytes that were depleted of CD34⁺ cells. As predicted, CD34 depleted CD56⁻, CD5⁻ TN thymocytes were no longer capable of reconstituting mFTOC (Fig. 10, E and F). However, the CD34⁻, CD56⁻, CD5⁻ TN thymocyte population maintained *in vitro* NK cell clonogenic capabilities. These results strongly support the contention that the majority of the CD56⁻, CD5⁻ TN thymocytes are NK cell precursors incapable of T cell differentiation, while the ability of T cell reconstitution of mFTOC is restricted to CD34⁺ TN thymocytes. The CD34⁺, CD56⁻, CD5⁻ TN cells, although capable of T cell development in mFTOC, showed no *in vitro* clonogenic potential using culture conditions optimized for growth of NK cells and mature T cells.

Discussion

In the present report, we have attempted to clarify the relationship between NK cells and T cells in the thymus by phenotypically dissecting human TN thymocytes on the basis of CD56, CD5, and CD34 surface antigen expression and determining the capacity of various subpopulations to generate NK cells in clonogenic culture conditions and T cells in thymic organ cultures. The CD5 antigen, although not restricted to the T cell lineage, was employed in our studies to identify thymocytes committed to the T cell lineage, since this antigen is not expressed on fetal or adult NK cells. By contrast most other T cell markers are shared between T and NK cells. For example, although not on thymic NK cells, CD28 is constitutively expressed on NK cells from fetal liver. In the thymus, both CD5 and CD28 antigens, however, are prominently expressed on CD34⁺ TN thymocytes capable of reconstituting thymic organ cultures (11, 12). CD56⁺, CD5⁻ TN thymocytes were present in all thymuses analyzed and comprised 3–15% of TN thymocytes. Phenotypically, the CD56⁺, CD5⁻ TN thymocytes were very similar to the fetal NK cells, including the expression of CD7 and variable expression of CD16. The CD16 antigen was not observed on a significant percentage of any other fetal or neonatal thymic population. Although thymic CD56⁺, CD5⁻ TN cells showed low levels of resting cytolytic activity, they demonstrated an NK cell clonogenic capability similar to that observed for fetal liver NK cells. Clones derived from the CD56⁺, CD5⁻ TN thymocytes expressed typical NK cell function and antigenic phenotype, including high surface density CD56, variable expression of CD16, and no surface CD3. As was observed in fetal liver-derived NK cell clones (17), a substantial proportion of these thymic NK clones expressed cytoplasmic CD3 proteins (data not shown), clearly indicating that detection of cytoplasmic CD3 proteins is no longer an unambiguous marker for the T cell lineage even in the thymus (17, 20). The CD56⁺, CD5⁻ TN thymocyte population, likewise, was unable to reconstitute mFTOC and thus con-

tained no T cell lineage-committed progenitors. The characteristics of the CD56⁺, CD5⁻ TN thymocyte population are consistent with the interpretation that these cells represent a relatively mature population of NK cells. In contrast to our findings, it was recently reported that a significant percentage of human CD3⁻, CD4⁻ thymocytes coexpressed the CD16 and CD1 antigens without expressing the CD56 antigen and that these cells could give rise to NK cell clones (14). We have been unable to verify these observations using TN thymocyte populations. In our preparations, CD16 was expressed primarily on NK cell-committed CD56⁺ cells. Since the CD56 antigen is expressed at relatively low surface density on fetal NK cells, these investigators probably failed to detect CD56 expression due to inadequately sensitive measurement techniques.

In addition to the CD56⁺, CD5⁻ TN thymocyte population, a significant percentage of TN thymocytes did not express either the CD56 or CD5 antigens. At first we assumed that these CD56⁻, CD5⁻ cells represented early T cell thymic progenitors. A careful analysis of this population, however, clearly indicated an antigenic phenotype strikingly similar to the CD56⁺, CD5⁻ TN population, including high surface density CD45 expression (a characteristic associated with mature lineage-committed cells (36)). Likewise, the majority of these cells did not express the progenitor cell-associated antigen, CD34 or the T cell-associated CD28 antigen. Since the CD56⁻, CD5⁻ TN thymocyte population showed no cytolytic activity and did not express the NK cell-associated markers CD56 or CD16, we hypothesized that these cells may represent a novel population of NK cell-committed precursors. Indeed, the majority of clones established from the CD56⁻, CD5⁻ TN thymocytes were typical NK cell clones acquiring high surface density CD56 and potent cytolytic activity. Although we have characterized the CD56⁻, CD5⁻ TN thymocyte population as containing NK cell precursors, based on the ability of these cells to acquire NK cell cytolytic function and antigenic phenotype, it is also possible that these cells may represent a unique subset of NK cells with unusual characteristics. We cannot exclude the possibility that this population may also contain other precursor cells capable of differentiation in appropriate conditions. Indeed, although the majority of the CD56⁻, CD5⁻ TN population did not express the progenitor cell-associated antigen CD34, a small percentage of CD34⁺ T cell progenitors were consistently observed within this population that could efficiently repopulate mFTOC and differentiate into CD3⁺, CD4⁺, CD8⁺ T cells. Removal of these CD34⁺ cells from CD56⁻, CD5⁻ TN thymocytes had no effect on the NK cell clonogenic potential of this population. Our results, in accordance with previous reports (12), clearly show that the expression of CD34 on TN thymocytes is primarily restricted to cells with T cell progenitor capabilities that do not possess *in vitro* clonogenic capability in IL-2 (Table 1). The growth and differentiation of T cell precursors requires the presence of thymic stromal cells as previously reported (12, 13, 37, 38, 39). Although NK cell and NK cell precursors are TN thymocyte populations distinct from the majority of T cell progenitors, there could exist a T/NK common pro-

Table 1. Human Thymic TN Populations

Characteristics	NK cell-committed thymocytes CD34 ⁻		T cell progenitor thymocytes CD34 ⁺	
	CD56 ⁺ ,CD5 ⁻	CD56 ⁻ ,CD5 ⁻	CD56 ⁻ ,CD5 ⁻	CD56 ⁻ ,CD5 ⁺
Antigenic phenotype	CD1 ⁻ ,CD28 ⁻ , CD45RA ⁺⁺ ,CD45 ⁺⁺	CD1 ⁻ ,CD28 ⁻ , CD45RA ⁺⁺ ,CD45 ⁺⁺	CD1 ⁻ ,CD28 ⁺ , CD45RA ⁺ ,CD45 ⁺	CD1 ⁺ ,CD28 ⁺ , CD45RA ⁻ ,CD45 ⁺
Lytic function (fresh)	YES	NO	NO	NO
NK cell clonogenic potential	YES	YES	NO	NO
Thymic reconstitution capability	NO	NO	YES	YES

- No surface expression; + dim surface density; ++ bright surface density.

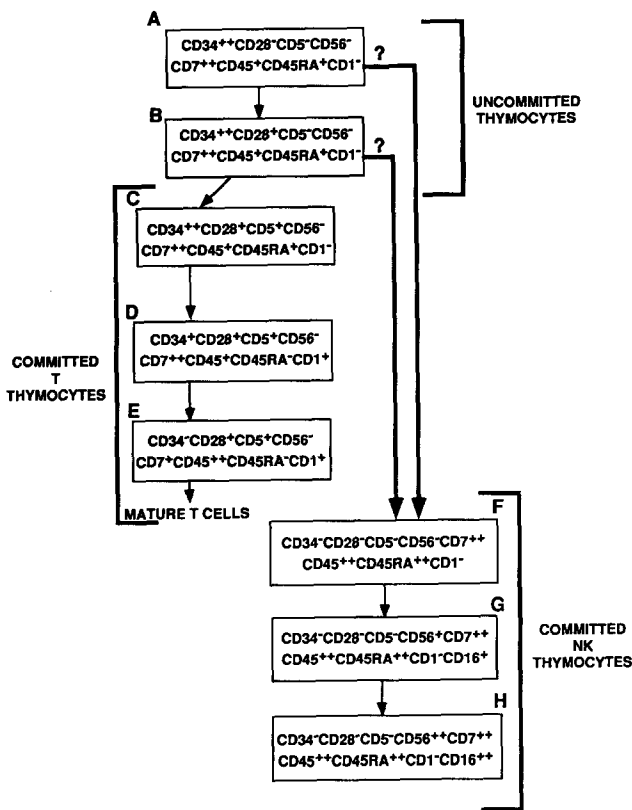


Figure 11. Hypothetical model of early human thymocytes development: a view from uncommitted thymocytes to mature T and NK cells. The hypothetical earliest thymic immigrant would express high density (++) of surface antigens CD34 and CD7, and lack the expression (-) of T and NK cell-associated surface markers (stage A). Under the influence of the thymic microenvironment, CD34⁺⁺ progenitors would differentiate towards a committed T cell lineage by sequentially expressing CD28, CD5, and CD1 and concomitantly down regulating (++) ⇒ + ⇒ (-)

genitor that is incapable of in vitro differentiation/maturation using our culture conditions. Indeed, a previous study (40) has shown that NK cells can be generated from bone marrow-derived CD34⁺ cells when cultured on appropriate stromal cell lines. Based on our observations and those of others (12, 41, 42), we are now able to propose a hypothetical model that may reflect the sequence of phenotypic and functional changes leading to the differentiation of NK cells and T cells in the thymus (Fig. 11). According to our hypothetical model, the most primitive hematopoietic progenitors to enter the thymus would phenotypically resemble bone marrow and fetal liver-derived hematopoietic progenitors (Fig. 11 A), expressing high surface density CD34 and CD7 antigens, but lacking the surface expression of CD5, CD28, CD56, and other lineage-associated antigens. As differentiation under the influence of the thymic microenvironment proceeds, CD28 (Fig. 11 B) and CD5 (Fig. 11 C) expression is induced on the high surface density CD34⁺ progenitor cells. Before the expression of CD5, we propose that the high surface density CD34⁺ progenitor cells are uncommitted to the T cell lineage and are thus capable of differentiation down the NK cell pathway, resulting in the sequential loss of CD34 and the gradual acquisition of CD56, CD16, and cytolytic function (Fig. 11, F-H). We speculate that the high surface density CD34-expressing progenitor cells that do not coexpress CD5 are the most likely candidates for a common T/NK progenitor population. Establishment of high efficiency in vitro cloning conditions that support the differentiation of NK cells from thymic CD34⁺ population are required before we

the CD34 antigen (stages B, C, D, and F). The uncommitted progenitor populations (stage A and/or B) could differentiate towards the NK lineage, resulting in the down regulation of CD34 and progressive expression of NK cell associated markers (stages F, G, and H).

can definitively identify the T/NK cell common progenitor. Our model would also predict that T cell differentiation would result in the progressive loss in CD34 expression on CD28⁺, CD5⁺ TN thymocytes and the concomitant expression of CD1 (Fig. 11 D). This sequence of differentiation events would thus signal irreversible commitment to the T cell lineage.

In conclusion, although it is generally thought that human TN thymocytes represent a relatively homogeneous population of T cell progenitors, our results indicate that they can be subdivided into two major populations: CD34⁻ cells that proliferate and generate NK cells in cloning culture conditions but are unable to differentiate to T cells in mFTOC; and CD34⁺ T cell progenitors that are unable to differentiate to NK cells in our culture conditions (Table 1). The

identification of an immature NK cell precursor population within the thymic microenvironment raises the question whether the thymus is indeed a site for NK cell differentiation. We have previously reported that NK cells mature early in human development, before the appearance of a thymic anlage (17). These studies together with the reports showing normal development of NK cells in athymic mice (16) would argue against the thymus as the only site of NK cell differentiation. Indeed, we have recently identified CD56⁻, CD5⁻, CD7⁺ NK cell precursor populations in most fetal hematopoietic organs, suggesting that NK cell differentiation is not restricted to any one tissue location. At present we do not know if thymic NK cells and NK cell precursors play a physiological role in normal T cell development.

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