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Human Thymic Epithelial Cells Inhibit IL-15- and IL-2-Driven Differentiation of NK Cells from the Early Human Thymic Progenitors

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T/NK progenitors are present in the thymus; however, the thymus predominantly promotes T cell development. In this study, we demonstrated that human thymic epithelial cells (TEC) inhibit NK cell development. Most ex vivo human thymocytes express CD1a, indicating that thymic progenitors are predominantly committed to the T cell lineage. In contrast, the CD1a+CD3−CD56+ NK population comprises only 0.2% (n = 7) of thymocytes. However, we observed increases in the percentage (20- to 25-fold) and absolute number (13- to 71-fold) of NK cells when thymocytes were cultured with mixtures of either IL-2, IL-7, and stem cell factor or IL-15, IL-7, and stem cell factor. TEC, when present in the cultures, inhibited the increases in the percentage (3- to 10-fold) and absolute number (3- to 25-fold) of NK cells. Furthermore, we show that TEC-derived soluble factors inhibit generation of NK-CFU and inhibit IL15- or IL2-driven NK cell differentiation from thymic CD34+ triple-negative thymocytes. The inhibitory activity was found to be associated with a 8,000- to 30,000 Da fraction. Thus, our data demonstrate that TEC inhibit NK cell development from T/NK CD34+ triple negative progenitors via soluble factor(s), suggesting that the human thymic microenvironment not only actively promotes T cell maturation but also controls the development of non-T lineage cells such as the NK lineage. *The Journal of Immunology, 2001, 166: 2194–2201.

The thymus is a lymphoid-epithelial organ that is essential for development of a majority of TCRαβ T cells in humans and mice (1–4). Early during embryonic development, progenitor cells from the yolk sac and fetal liver colonize the thymic anlage where they undergo sequential developmental stages that can be identified by the differential expression of specific cell surface molecules (5, 6). In humans, intrathymic progenitor cells, identified by the CD34+CD38low phenotype, have recently been shown to have bipotential differentiation ability and, under the appropriate conditions, can differentiate into either T cells or NK cells (7–10). The human T/NK bipotential progenitors have been further characterized as CD1a+CD5−CD3−CD4+CD8−CD7+ (9). In mice, thymic T/NK precursors are NK1.1+CD117+CD44−CD25− (11); a large portion of these cells express CD122 and IL-15Rα (12, 13). Recently, it has been demonstrated at the single-cell level that early thymic progenitors indeed have the potential to differentiate into either T or NK cells (14, 15). T cell development in the thymus generally can be followed by the expression of CD3, CD4, and CD8 surface markers. Thus, the most immature cells are CD3−CD4−CD8− (triple negative, NT3) and will differentiate into CD3−CD4+/−CD8−, CD3+/−CD4+CD8+ (double positive, DP) and CD3+CD4+ or CD3+CD8+ mature T cells (16, 17). In humans, expression of CD1a by thymic precursors indicates T lineage commitment (18, 19).

Although the early thymic progenitors have the capacity to differentiate into either T or NK cells, the thymic microenvironment predominantly supports T lineage commitment and differentiation, such that only a very small percentage of NK cells is present in the thymus (10, 11, 13). The development of the majority of NK cells occurs outside the thymus, in the bone marrow (11, 20, 21). Furthermore, it is established that the development of NK cells is thymus-independent because NK cells are detectable in embryonic liver as early as 6 wk of gestation before formation of the thymic anlage (22), and NK cells are present in athymic nude mice (23, 24). Thus, although it is clear that the thymus contains NK precursors, it is not known why the vast majority of T/NK progenitor cells in the thymus commit to the T lineage and only a very small percentage of T/NK cells commit to NK differentiation and maturation within the thymic microenvironment (7, 25). Whether the thymic microenvironment, particularly thymic epithelial cells, regulates the development of NK cells within the thymus or influences the preferential T lineage commitment of the T/NK bipotential progenitors has not been investigated.

It has been established that IL-2 and particularly IL-15 can induce NK differentiation from bone marrow or thymic progenitors in both humans and mice (13, 26–29). Furthermore, IL-7 and stem cell factor (SCF)3 can synergize with IL-2 and IL-15 to induce NK differentiation (26, 30). In this study, we tested whether primary cultured human thymic epithelial cells (TEC) or various molecular mass fractions of their culture supernatants affect cytokine-driven differentiation of

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3 Abbreviations used in this paper: SCF, stem cell factor; TEC, thymic epithelial cell; TES, thymic epithelial cell culture supernatant; BM, bone marrow; b-HLH, basic helix-loop-helix.
CD3+ CD56+ NK cells from total human thymocytes and CD34+-enriched TN human thymocytes (CD34+ TN).

Materials and Methods

Antibodies

The following Abs were used in this study: anti-CD1a (OKT-6), anti-CD2 (35.1), anti-CD3 (OKT-3), anti-CD4 (OKT-4), and anti-CD8 (51.1) (American Type Culture Collection, Manassas, VA); anti-CD1a-CyChromeg (Cy-Chr; HH149), anti-CD56-PE (B159), anti-CD3-Chr (UTCHT1), anti-CD34-APC (581), and anti-CD38-FITC (HT2) (PharMingen, San Diego, CA); anti-NKR-P1A (DX12, HP3G10, 191B8), anti-p58.1 (HP3E4, EB6), anti-p58.2 (CHL, GL183), anti-CD94 (HP3B1, XA185, HP3D9) (Leuco-cyte Typing VI); and goat anti-IgG (H + L)-FITC (human serum adsorbed) (Life Technologies, Gaithersburg, MD). All Abs were titrated to obtain optimal concentrations for flow cytometric analyses.

Human TEC cultures

Normal human thymi were obtained as discarded tissues through the Department of Cardiovascular Surgery and Pathology, Loyola University Medical Center from otherwise healthy children <1 year of age undergoing corrective cardiovascular procedures for congenital heart diseases. TEC cultures were initiated by an explant technique and propagated in supplemented medium as previously described (31). Contaminating thymic fibroblasts were removed by treatment with 0.02% EDTA, followed by anti-fibroblast mAb (1B10) treatment, and complement-mediated cell lysis (32). TEC cultures were monitored for contaminating macrophages and fibroblasts using Mo-1 and TE-7 mAb, respectively (American Type Culture Collection). Human thymic thymocytes were used for each enrichment separation. Freshly obtained human thymocytes were enriched for keratin as determined by reactivity to anti-keratin mAb NMF-116 (Dako, Carpinteria, CA).

Thymocyte cultures

Human thymocytes were gently teased from thymus tissue, isolated by Ficoll-Hypaque centrifugation, and slowly frozen in RPMI 1640 (Mediatech, Herndon, VA) containing 20% FCS (Life Technologies), 7.5% DMSO (Sigma, St. Louis), and 10 μg/ml gentamicin (Life Technologies). Frozen thymocytes were thawed quickly and incubated with RPMI 1640 containing 30% FCS, 100 μg/ml DNase I (Sigma), and 10 μg/ml gentamicin for 1 h at 37°C. Dead cells were removed by Ficoll-Hypaque centrifugation. In some experiments, thymocytes were used immediately after isolation from thymus tissue without freezing. Thymocytes were cultured in RPMI 1640 supplemented with 10% FCS (Life Technologies), 10% human pooled AB serum (Sigma), and 10 μg/ml gentamicin. For coculturing with TEC, thymocytes were placed in TEC culture chambers (0.2 mm; Num Naige, Naperville, IL) which were then placed in six-well cluster dishes previously plated with TEC. Thymocytes at 3.0 × 106 cells were cultured with 5 × 103 TEC in a total volume of 3.0 ml/well. The following cytokines were used: human rIL-2 (100 U/ml); Cetus, Emeryville, CA); IL-7 (10 ng/ml), SCF (10 ng/ml; BioSource International, Camarillo, CA); and IL-15 (10 ng/ml; R&D Systems, Minneapolis, MN). Human 3T3 fibroblasts (35.1) were used at 5 ng/ml. The cells were cultured with IL-2 or IL-7 alone, or with combinations of IL-2, IL-15, and SCF. Thymocytes were removed, washed, adjusted to equal cell density, and cultured with newly plated TEC every 5–7 days. Thymocytes were cultured up to 30 days and were harvested at various time points and assayed for cell surface markers by flow cytometry as described elsewhere (33).

Culture of CD34+-enriched TN thymocytes

CD34+-enriched TN thymocytes were isolated from freshly obtained thymocytes (not frozen) using the negative selection StemSep kit (Stem Cell Technology, Vancouver, Canada). Between 5 × 106 and 2 × 107 thymocytes were used for each enrichment separation. Freshly obtained human thymocytes were first incubated with anti-CD1a, anti-CD3 mAbs. The cells were then treated with goat anti-mouse Ab coupled to magnetic colloidal at a ratio of 80 particles/cell (BioMag; Perceptive BioSystems, Framingham, MA) for 30 min at 4°C. The CD1a+ and CD3+ cells were then removed using a strong magnetic plate. This step was repeated twice or was repeated until the cell suspension was clear of magnetic colloidal particles. The unbound cellular fraction containing predominantly the CD1a+ CD3+ cells was then collected and subjected to a second round of negative selection using the StemSep system. In the second step, the cells were treated with a mixture of anti-CD3 and anti-CD1a mAbs and were added to CD3+ Abs to CD2, CD4, CD8, CD19, CD24, CD56, CD66, and glycoporphin A (StemSep) for 30 min on ice. A total of 2 μl of the Ab mixture was used for each 1 × 107 cells as suggested by the manufacturer. After incubation with magnetic colloid (60 μl/1.0 ml of cell suspension) for 30 min, the cells were loaded into a magnetic column and washed with 8 ml of medium according to the manufacturer’s protocol. The flow-through fraction containing lineage-negative cells enriched for CD34+ cells was collected. Purity of the isolated cell fraction was determined by flow cytometric analysis using CD34-APC, CD38-FITC, CD3-PerCP, CD56-PE, and CD1a-PE. The isolated cells (1 × 103 cells/well) were cultured in 96 U-bottom well culture dishes in RPMI 1640 plus 10% FCS and 10% human AB serum. The following combination of cytokines were included: IL-2 (100 U/ml) + IL-7 (10 ng/ml) + SCF (10 ng/ml) and IL-15 (10 ng/ml) + IL-7 + SCF. In the coculture system, TEC were plated onto transwells at 1.5 × 103 cell/well. Flow cytometric analysis was performed at day 8 or 12. We also cultured CD34+-enriched TN thymocytes with cytokines in the presence of two molecular mass fractions (8,000–30,000 Da, >30,000 Da) isolated from TEC culture supernatants.

Fractionation of TEC culture supernatants

Culture supernatants from confluent cultures of human TEC were collected every 3 days. The pooled supernatants were first clarified by centrifugation and then filtered through a 0.2-μm filter. A total of 160 ml of culture supernatants was concentrated using Centricron Plus-80 centrifugal filter devices (Millipore, Bedford, MA). The supernatants were first filtered through a 30,000-Da cutoff and the filtrates were then filtered through an 8,000-Da cutoff filter. The molecular mass fractions were designated as >30,000 and 8,000–30,000 Da, respectively. The fractions were tested at various final concentrations.

NK cytotoxicity assay

Measurement of NK cell lytic activity for K562 and Daudi cells was performed as previously described (34). Briefly, 1 × 106 K562 and Daudi cells were labeled with 100 μCi of 51Cr. Sodium carbonate for 1 h at 37°C. Labeled target tumor cells were cultured with effector cells at various E:T ratios for 4 h. The supernatants were removed using a Skatron harvesting press (Skatron, Sterling, VA) and associated radioactivity determined with a 4600 ME PLUS automated gamma counter. Maximum release of radioactivity was obtained by adding 0.05% Nonidet P-40 (Sigma). Lytic activity was expressed as percent cytotoxicity as calculated by the formula: percent cytotoxicity = ([experiment dpm] – [minimum dpm])/[(maximum dpm) – (minimum DPM)] × 100. All experimental means were calculated from triplicate values.

NK-CFU colony-forming assay

NK colony assays were performed according to a method previously described by Ogawa and colleagues (35). Briefly, postnatal CD34+ TN thymocytes were cultured at 50 × 103 and 100 × 103 cells in 35-mm vented lid suspension culture dishes (Nalgene). The cells were suspended in 1.0 ml of Iscove’s medium (Mediatech) containing 1.2% methylcellulose (Stem Cell Technology), 10% FCS, 10% human AB serum, 10 μM pénicillin-streptomycin, and 5 × 10−3 M 2-ME. The cells were cultured with a combination of IL-2 (2 U/ml), IL-7 (10 ng/ml), and SCF (10 ng/ml) × IL-15 (10 ng/ml), IL-7, and SCF. The cells were cultured for 20 days at 37°C in a humidified atmosphere with 5% CO2/95% air. The NK-CFU were identified by their large, diffuse morphology as previously described and confirmed by Ogawa and colleagues (35). The colonies were counted and the average number of colonies per duplicate well was determined. To confirm NK phenotype, single colonies were plugged and stained with Wright stain (New Comer Supply, Middleton, WI) to detect cytoplasmic granules.

Flow cytometric analysis

Cells were harvested and washed twice in PBS/2% BSA. Two hundred thousand cells (2 × 105) were stained and analyzed by flow cytometry using a FACScanlibur (Becton Dickinson, San Jose, CA). For three-color analysis, cells were stained with each mAb for 30 min on ice and washed three times with PBS/2% BSA. If nonconjugated mAbs were used, cells were first stained with these mAbs followed by washing and incubation with goat anti-mouse Ig (H + L)-FITC (human serum adsorbed). Data analyses were performed with CellQuest (Becton Dickinson) or WinMDI version 2.8 (Scripps Research Institute, San Diego, CA). Appropriate isotype control Abs were used to set markers where >98% of the cells are negative.

RT-PCR

Cells were lysed in 4 M guanidine isothiocyanate and total RNA was isolated by centrifugation through a 5.7 M cesium chloride gradient as previously described (36). Alternatively, mRNA was purified using the QuickPrep Micro mRNA purification kit (Pharmacia, Piscataway, NJ). Synthesis of first-strand cDNA was performed with 3 μg of total RNA or...
100 ng of mRNA using a first-strand synthesis kit (Pharmacia) according to the manufacturer’s instructions. The PCR amplification reactions contain 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.2 mM MgCl₂, 0.125 mM dNTP, 0.25 µM primers, and 0.025 U/µl Taq (Life Technologies). For IL-7, two rounds of PCR amplification were performed: the first-round PCR was performed with 2 µl of cDNA samples for 25 cycles, and the second-round PCR was performed with 3 µl of the first-round PCR products for 30 cycles. IL-15 and GAPDH were performed with one round of 35 cycles of amplification. Each PCR cycle includes 15 s at 94°C, 20 s at 55°C, and 40 s at 72°C; the last cycle was followed by a final 7-min extension step at 72°C. PCR were performed with a GeneAmp PCR 2400 instrument (Perkin-Elmer/Cetus, Norwalk, CT). PCR products were separated in 2% agarose gel and visualized with ethidium bromide staining. Gel images were captured using a Kodak digital camera with one-dimensional image analysis software (version 2.1.; Kodak, Rochester, NY). Primer sequences for the amplified regions were as follows: IL-7: forward, 5'-atg gcaaac aat atg gaa-3'; reverse, 5'-ttt cga aac cac att tga gaa-3'.

**Results**

Most ex vivo human thymocytes from postnatal thymus are committed to the T cell lineage and only a fraction of thymocytes is NK phenotype.

Because the T/NK bipotential precursors were found in the human thymus (8, 10, 37), we determined whether the thymus affects NK development in vivo. Most ex vivo human thymocytes from postnatal thymus are CD1a+CD3-CD56+ NK cells, we wanted to determine whether TEC affect NK cell development. Total thymocytes were cultured with a combination of IL-15 (10 ng/ml), IL-7 (10 ng/ml), and SCF (10 ng/ml) and were analyzed for expression of CD3 and CD56 by two-color flow cytometric analysis. The combination of the above cytokines has been shown to optimize the induction of NK differentiation from thymic precursors in vitro (8). Increased percentages of CD3+CD56+ cells were observed with increasing culture periods (Fig. 3). In addition to the increases in the percentage of CD3+CD56+ cells, we also observed increases in the percentage of CD3+CD56+ cells and a concomitant decrease in the percentage of CD3+CD56+ cells; the percentage of CD3+CD56- cells remained unchanged (Fig. 3). In contrast, TEC, when cultured with thymocytes in transwells, inhibited the in vitro development of CD3+CD56+ cells (Fig. 3). The decreases in the percentage of CD3+CD56- cells were associated with increases in the percentage of CD3+CD56- T cells and CD3-CD56+ cells (Fig. 3). TEC had no effect on the development of CD3+CD56- cells (Fig. 3). Similar results were obtained when thymocytes were cultured with IL-2, IL-7, and SCF in the presence of TEC (data not shown). To confirm that the inhibition of CD3+CD56+ cell development by TEC was not due to selective TEC-induced outgrowth of other subsets, we determined the absolute number of CD3+CD56+ cells. Fig. 4 shows results from six independent experiments in which cells were cultured with either IL-2, IL-7, and SCF (left panels) or IL-15, IL-7, and SCF (right panels) in the presence or absence of TEC. We demonstrated that the number of NK cells increased with time when thymocytes were cultured with mixtures of cytokines in the absence of TEC. In contrast, a dramatic reduction of NK cell numbers was observed when TEC were present in thymocyte cultures. We observed a 6- to 12-fold reduction in the number of CD3+CD56+ cells in thymocyte cultures treated with IL-2, IL-7, and SCF, and a 3- to 25-fold reduction in cultures treated with IL-15, IL-7, and SCF (Fig. 4). We also determined that the absolute number of cells in the CD3+CD56- and CD3+CD56- subsets were not affected by

**FIGURE 1.** Phenotypes of CD56-positive ex vivo human thymocytes.

Three-color flow cytometric analysis was used to determine the phenotypes of CD56-positive ex vivo human thymocytes. Thymocytes were stained with anti-CD3-FITC, anti-CD56-PE, and anti-CD1a-CyChr and analyzed by flow cytometry. A, Forward light scatter and side light scatter profile; R1 depicted the analyzed thymocyte population; B, CD1a-CyChr, CD3-FITC; C, CD56-PE, CD3-FITC; D, CD56-PE, CD1a-CyChr gated on thymocytes (R1) and CD56- (R3); E, CD56-PE, CD1a-CyChr; F, CD56-PE, CD3-FITC gated on thymocytes (R1) and CD56- (R2). One representative result of six experiments performed with thymocytes from six different human pediatric thymi.

<table>
<thead>
<tr>
<th>% of Positive Cells Within Each Subset</th>
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<tr>
<td>CD3+CD56+</td>
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<td>2.3 ± 1.1*</td>
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*Percentage of positive cells as determined by three-color flow cytometric analysis. Data represent the average values plus SE calculated from seven experiments performed with human thymocytes that were obtained from seven different pediatric thymi.
TEC; a moderate increase in the absolute number of CD3\(^+\)CD56\(^-\) cells however were observed (data not shown).

**TEC also inhibits NK cell development from the CD34\(^+\)-enriched CD3\(^-\)CD4\(^-\)CD8\(^-\) TN thymocytes**

To differentiate between mature NK expansion and differentiation of NK progenitor cells, we next determined whether TEC inhibit NK development from the isolated CD34\(^+\) TN progenitors. As previously shown by others (9, 10), we determined that the human CD34\(^+\)-enriched TN thymocytes can differentiate into functional NK cells in our culture system. The ex vivo-enriched progenitor population was negative for CD3 and CD56 (Fig. 5A). The CD34\(^+\)-enriched TN cells were positive for CD38 and CD7, but were negative for the early T cell marker CD1a (data not shown). Further phenotypic analysis showed that >60% of CD1a\(^-\) CD3\(^-\)CD56\(^+\) cells express CD94 and the NK cell marker NKRP1A (CD161); however, the cells do not express the killer inhibitory receptor receptors p58.1 (HP3E4, EB6) and p58.2 (CHL, GL 183) (Fig. 5, D–I). Functionally, the thymic-derived CD3\(^-\)CD56\(^+\) NK cells display potent cytotoxicity for both K562 and Daudi target cells (Fig. 6). Thus, we could generate functionally mature NK cells in vitro from the human CD34\(^+\)-enriched TN thymocytes. Similar results were obtained with IL-15, IL-7, and SCF (data not shown). This in vitro culture system thus provides a functional model where regulation of NK differentiation can be investigated. We next examined whether TEC regulate NK differentiation from the CD34\(^+\)-enriched TN thymocytes. As observed with total thymocyte cultures, TEC also inhibited the differentiation of CD3\(^-\)CD56\(^+\) cells from the early thymic progenitor cells; while 85% of CD3\(^-\)CD56\(^+\) cells were detected in the absence of TEC, only 42% of CD56\(^+\) cells were detected in the presence of TEC (Fig. 7, top three panels). In a separate series of experiments, a 8,000- to 30,000-Da fraction derived from TES inhibits NK differentiation from thymic progenitors (Fig. 7, bottom four panels).

**FIGURE 2.** Expression of IL-15 and IL-7 mRNA by human TEC. IL-15 and IL-7 mRNA were detected by RT-PCR. GAPDH was used to monitor the quality of the isolated mRNA and cDNA synthesis. Data were from five different TEC cultures generated from five different human pediatric thymi.

**FIGURE 3.** Human TEC inhibit the development of CD3\(^-\)CD56\(^+\) NK cells from total human thymocytes. Thymocytes were cultured with IL-15 (10 ng/ml), IL-7 (10 ng/ml), and SCF (10 ng/ml) in the presence or absence of human TEC as described in Materials and Methods. Thymocytes were harvested at days 5, 12, and 19, and 2.0 \(\times\) 10\(^5\) cells were analyzed for the expression of CD3 and CD56 by flow cytometric analysis. At days 5 and 12, cells were adjusted to an equal cell number and cultured until the next indicated time points. Results from one representative experiment of six experiments performed with thymocytes isolated from six pediatric thymi.
Although we noticed that the inhibition was not as completed as in the case of total thymocytes (Figs. 3 and 4), the consistent results from several independent experiments strongly indicate that TEC do regulate NK cell development. IL-7 and SCF have been shown to synergize with IL-2 or IL-15 in the induction of NK cell differentiation (26, 30). However, IL-15 or IL-2 themselves can induce NK differentiation from thymic and bone marrow precursors. Thus, we determined whether TEC directly inhibit IL-15- or IL-2-driven NK cell development. We showed that TEC or the 8,000- to 30,000-Da fraction are potent inhibitors of IL-15- or IL-2-driven NK cell development from the CD34⁺ TN progenitors; the >30,000 Da showed no inhibitory effect (Fig. 8). Furthermore, the development of both the CD3⁻CD56⁺ and CD3⁻CD56⁻ cells was dramatically reduced by TEC or by the TEC-derived 8,000- to 30,000-Da fraction (Fig. 8). Since TGF-β has been shown to inhibit NK development from murine bone marrow (BM) (38) and we have shown that human TEC produce active TGF-β (39), we tested whether TGF-β affects NK development from thymocytes. Fig. 9 shows that TGF-β has no effect on cytokine-driven NK development.

We also performed NK colony assays to determine the effect of TEC on NK cell development at the single precursor cell level. As shown in Table II, TES served as a potent inhibitor of NK-CFU when CD34⁺ TN thymocytes were cultured with IL-2, IL-7, and SCF or IL-15, IL-7, and SCF; however, TES did not affect GM-CFU formation. Thus, TEC specifically inhibit the development of NK-CFU from thymic CD34⁺ TN progenitors.

**Discussion**

In this paper, we present data describing a novel regulatory function of human TEC in the development of NK cells from the early thymic progenitors. TEC, through signaling via adhesion molecules and cytokines, have been shown to play a critical role in supporting the development of TCRab T cells (1, 3, 40); our data indicate that TEC also regulate NK cell development from the early human thymic precursors.

Recently, it has been established at a single-cell level that a subset of the TN thymocytes is bipotential and can differentiate into either T or NK cells in vitro (14, 15). Despite this, a majority of these cells develop into T cells, suggesting the thymic microenvironment controls the outcome of this process. Züniger-Pflücker and colleagues (11) have demonstrated that exposure of T/NK progenitors to a thymic microenvironment results in a predominant commitment to the T lineage, whereas culture of bipotential cells with BM-derived-stroma promotes the generation of NK cells. Plum and colleagues (13) have suggested that the differential requirements for stroma cell-precursor cell interaction and/or cytokines directs differentiation toward either T or NK cells from T/NK progenitors. In vivo studies with IL-15- and IL-15Ra-deficient mice indicate that IL-15 plays an obligatory role for NK cell differentiation and functional maturation; lack of NK cells in BM and thymus were observed in these mice whereas the development of NK cells in thymus was unaffected.
of thymus-dependent T cells is intact (41, 42). IL-2 has also been shown to induce NK cell development in vitro; however, NK development proceeds normally in IL-2-deficient mice, confirming the essential role of IL-15 in the differentiation of NK cells in vivo (43). In addition to IL-15 and IL-2, other cytokines such as IL-7, SCF, and Flt3 ligand can synergize with IL-15 or IL-2 to induce NK cell development. However, IL-7, SCF, and Flt3 ligand by themselves are not able to induce NK cell differentiation, but mediate the IL-15 response by regulating IL-15Rα expression (38, 44, 45). In contrast, IL-7 is critical for TCRab T cell development (46) and high concentrations of IL-15 (500 ng/ml) have been shown to inhibit the development of TCRαβ T cells in murine fetal thymic organ culture (13). Taken together, previous work identifies critical cytokines for T cell and NK cell development and supports the notion that differential requirement of cytokines affects the differentiation of T/NK bipotential cells toward either T cell or NK cell lineages. The in vitro data with FOTC also support the notion that differentiation of NK cells in the thymus is regulated, and egression of mature NK cells from the thymus is unlikely to account for the low percentage of NK in the thymus. However, direct evidence showing the control of NK development in the thymus is lacking.

The paucity of intrathymic NK cells may result from thymic microenvironment-mediated differential responses to cytokines present in the thymus. Thus, controlling IL-15-driven NK differentiation from thymic progenitors can regulate intrathymic NK development. It is unlikely that lack of IL-15 in the thymus is the cause for the low percentage of intrathymic NK cells because IL-15 is expressed in murine thymic stroma cells (13) and we showed here that human TEC express IL-15.

Although the CD3−CD1a−CD56+ NK cells comprise only 0.2% of total thymocytes in the thymus, dramatic increases in the percentage and the absolute number of CD3−CD56+ NK cells were obtained with thymocytes cultured with cytokines. Because we observed increases in both the percentage and absolute cell number of NK cells, the increases in NK cells were not due to selective depletion of other thymocyte subsets. Indeed, we have previously reported that TEC induce TN thymocyte proliferation (47). The inhibition was not caused by cell death of NK precursors or committed NK cells because, upon removal of TEC from the cocultures, we again detected increases in the percentage and absolute number of CD3−CD56+ cells after an additional 18 days in culture (data not shown). Furthermore, because we observed a reduction in the absolute number of NK cells, we can exclude the possibility that the inhibition was caused by the TEC-mediated selective proliferation of other T cell subsets. Although our data indicate that TEC regulate the outgrowth of pre-existing thymic cells with a NK phenotype, they do not exclude the possibility that differentiation of NK cells from thymic progenitors is also regulated by TEC.

To test whether TEC directly regulate NK differentiation, we determined the effect of TEC on NK cell differentiation from human CD34+CD1a−TN thymocytes. The isolated CD34+ TN thymocytes are CD38+ and CD7+, but are CD1a−, characteristic of the previously reported human T/NK thymic progenitors (8, 10); this isolated population contains no CD3−CD1a−CD56+ thymic NK cells. We showed that IL-15- and IL-2-driven differentiation of NK cells was inhibited by TEC-derived soluble factors. The inhibitory effect with the CD34+ TN thymocytes was not as complete as in the case of total thymocytes; however, we observed a consistent inhibition of NK differentiation in a series of three independent experiments. Perhaps, the presence of the developing thymocytes also contributes to the overall inhibition of NK cell differentiation. We did not detect CD3−, TCRαβ+, and TCRγδ+ T cells or CD83+ dendritic cells in our cultures. This was not
range encompasses most of the cytokines currently known to be expressed by human TEC (33). Currently, a specific cytokine that can inhibit IL-2- or IL-15-driven NK cell differentiation has not been identified. Caligiuri and colleagues (38) have shown that TNF-α and TGF-β inhibit NK cell development from BM precursor cells in the presence of IL-15 and Flt-3 ligand. Our data indicate that TGF-β3, a predominant TGF-β isoform in our TEC cultures (39), does not inhibit cytokine-driven NK cell development.

Recent emerging data at the molecular level have provided new insight for understanding the control of NK cell development. Transcription factors with a basic helix-loop-helix (b-HLH) motif are well established as regulators of cellular differentiation (50). Id, a subfamily of the b-HLH transcription family, functions as a natural dominant negative regulator of the b-HLH transcription factor activity (51). Mice deficient in Id2 fail to generate thymic-, spleen-, and BM-derived NK cells (52). The NK cell developmental defect cannot be rescued with IL-15, indicating that this is an intrinsic defect rather than microenvironment and that expression of Id2 is required for IL15-driven differentiation of NK cells (52). In contrast, overexpression of Id2 in Id2-transgenic mice blocks early T cell development (53). Similarly, forced expression of Id3, while inhibiting TCRαβ T cell development, promotes NK cell development from the T/NK bipotential precursor cells (54). From these findings, it is reasonable to speculate that TEC regulate NK development by controlling expression and activity of b-HLH transcription factors. Because b-HLH transcription activity is also important for T cell development (53, 55–57), regulation of their activities by TEC would affect both T and NK cell development in the thymus, particularly at the T/NK bipotential progenitor stage.

### References


