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Ontogeny of Thymic NK1.1+ Cells

Zuhair K. Ballas, Wendy L. Rasmussen, Carol A. Alber, and Matyas Sandor

Thymic NK1.1+ cells are a recently described lymphocyte subset whose biologic function is not well defined. There is some controversy as to whether thymic NK1.1+ cells mature in a thymic or an extrathymic pathway. In this study, we examined the ontogeny of murine thymic NK1.1+ cells utilizing direct examination of freshly obtained fetal thymi as well as fetal thymi established in organ cultures (FTOC). We found a reproducible peak (5-40%) of NK1.1+ cells, demonstrable in day 15 to 16 freshly obtained fetal thymi, which was markedly decreased by day 17 of gestation; this peak preceded the appearance of the CD4+CD8- thymocytes by 12 to 24 h. Reverse-transcriptase PCR analysis of NK1.1 demonstrated its presence as early as day 9 of gestation, thus placing it as one of the earliest lymphocytic genes to be transcribed. Utilizing FTOC, we found that: 1) day 12 fetal thymus contained a progenitor that can differentiate into an NK1.1+CD4+CD8- lymphocyte; 2) NK1.1+ cells dwindle to <5% in FTOC established from day 14 thymi; 3) NK1.1+ cells dominate in FTOC supplemented with IL-2; and 4) most of the NK1.1+ cells seen in FTOC did not express CD3e on their surface, except for the FTOC supplemented with IL-12. These findings suggest that NK1.1+ cells may play an important role in thymic maturation. Moreover, these findings suggest that fetal thymus contain several novel lymphocyte subsets that can be induced to overgrow the normal thymocytes upon exposure to certain cytokines.

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Materials and Methods

Mice

Four- to six-week-old, virus-free C57BL/6 breeding mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Timed pregnant mice were obtained in our animal facility; the time the vaginal plug was demonstrated was counted as day 0 of gestation. Some timed pregnant mice were purchased from Simonsen Laboratories (Gilroy, CA) or from Harlan Sprague Dawley (Indianapolis, IN).

Fetal thymic organ cultures

Fetuses were obtained at various days of gestation, and their thymi were removed using a surgical microscope and jeweler's fine forceps. Extreme care was taken to obtain whole intact lobes; damaged or partial lobes were not used. The thymic lobes were placed on an extensively boiled, ethylene oxide-sterilized, 0.4-μm Nucleopore filter, 13 mm in diameter. Each filter was placed on top of pretreated sterile Gelfoam surgical sponge (The Upjohn Co., Kalamazoo, MI) cut to fit a 35 × 10-mm tissue culture petri dish. Four thymic lobes were cultured per petri dish. Each spleen/fetal thymus set was bathed in Iscove's modified Dulbecco's medium (Life Technologies, Grand Island, NY) supplemented with 5 × 10^-5 M of 2-ME, non-essential amino acids, 5% FBS, and antibiotics. The culture medium was changed every 3 to 4 days. On the day of the harvest, the thymic lobes were pushed off the filter into test tubes, ground into a single cell suspension, washed three times in PBS supplemented with 1% BSA, and used for flow cytometry staining or as effectors in a 3Cr release assay. In some experiments, the culture medium was supplemented with certain cytokines: rIL-2 (Proleukin; Chiron Therapeutics, Emeryville, CA) was used at 1000 U/ml; mouse rIL-7 was a generous gift from Immunex (Seattle, WA) and was used at 100 ng/ml; and mouse rIL-12 was purchased from PharMingen (San Diego, CA) and was used at 1 ng/ml.

Flow cytometry

Surface markers were assessed by flow cytometry using three- and four-color analysis, as previously described (1, 12). The various Abs used were obtained from PharMingen, or were labeled in our laboratory from hybridomas obtained from American Type Culture Collection (Rockville, MD). Cells were analyzed using an EPICS 753 (Coulter Immunology, Hialeah, FL) instrument fitted with a logarithmic amplifier. All of the plots presented in this work are on a logarithmic scale. Software gates were set, and analysis was done using the Elite software. The quadrants in each presented plot were determined by the staining obtained using isotype controls.

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Polymerase chain reaction

mRNA was isolated using MicroFast Track mRNA Isolation Kit (Invitrogen Corp., San Diego, CA). cDNA was synthesized using Moloney murine leukemia virus (MMLV) reverse transcriptase (20 U/μl). PCR amplification was conducted using 5 μl of cDNA added to a reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates, 1 μM of each primer, and 2.5 U Taq polymerase (Boehringer Mannheim Corp., Indianapolis, IN). Each sample was overlaid with 50 μl of light mineral oil and incubated in a DNA thermal cycler for a total of 30 cycles. Each cycle consisted of 1 min at 95℃, 2 min at 60℃, and 1 min at 72℃. Each cDNA was also tested by amplification of β-actin message with specific primers. The NK1.1-specific primers were selected from the published sequence (13). The primers used to detect the NK1.1 message as a 428-bp product were: 5′-GCTCTGAAGCTCAGCTGTGCTG-3′ and 5′-GCCATTTTCAGTGACA CCAGTG-5′. PCR product analysis was done in a 2.5% agarose gel. Each sample was overlaid with 50 μl of light mineral oil and incubated in a DNA thermal cycler for a total of 30 cycles. Each cycle consisted of 1 min at 95℃, 2 min at 60℃, and 1 min at 72℃. Each cDNA was also tested by amplification of β-actin message with specific primers: 5′-TACTCCATCAGACAGTCCA-5′ and 5′-ACCTGACA GACTACCCTACA-3′ (14).

Results

NK1.1+ peak in day 15 to 16 fetal thymi

Fetal thymi were obtained from timed pregnant mice from day 12 through 19 of gestation, rendered into single cell suspension, and examined for their surface expression of NK1.1, CD3ε, CD4, and CD8. The data presented in Figure 1 clearly demonstrate a peak of NK1.1+ cells present in day 15 to 16 fetal thymi that were markedly decreased by day 17 of gestation. In more than 10 experiments of this kind, we found an NK1.1+ peak accounting for 5 to 40% of fetal thymi on day 15 to 16 of gestation. In general, this peak preceded the appearance of the CD4+CD8+ thymocytes by 12 to 24 h. None of the NK1.1+ cells expressed CD4 or CD8 on their surface (data not shown). The NK1.1+ cells did not express CD3ε (Fig. 1) or TCR-β (data not shown) on their surface. In data not shown, these NK1.1+ cells also expressed the following markers on their surface: 2B4, the β-chain of the IL-2R, CD2, and Thy-1.2; 50% expressed Ly6c. In addition, they were Ly49A-, Ly49C-, CD5−, L-selectin−, CD25−, CD24−, and CD45RB−. A trivial explanation for the disappearance of the NK1.1+ thymocytes is that they are overgrown. While a dilutional effect may be involved, we do not believe this to be the sole explanation. In general, the number of recoverable thymocytes triples or, at most, quadruples from day 16 to 17 of gestation, while the number of NK1.1 cells is reduced by close to tenfold.

Another explanation for the disappearance of the NK1.1+ thymocytes just as the CD4+CD8+ cells appear is that the NK1.1+ cells give rise to the CD4+CD8+ cells. However, in several experiments, we were unable to identify a significant number of NK1.1+CD4+CD8+ cells in fetal thymocytes obtained on day 12 to 19 of gestation (data not shown). It appeared possible that such a triple-positive population might exist, but that either NK1.1 surface expression is quickly down-modulated or such a population is quickly overgrown by continually seeding thymic progenitors. To address this issue, we obtained fetal thymi at days 12, 14, and 16 of gestation and from newborn mice. The intact thymic lobes were established in fetal organ cultures (FTOC) for 7 days, after which the thymic lobes were retrieved, and single cell preparations were obtained and examined for their surface expression of NK1.1, CD4, and CD8. As shown in Figure 2, day 12 fetal thymi gave rise to the usual four populations of thymocytes: CD4+CD8−, CD4+CD8+, CD4−CD8+, and CD4−CD8+. Interestingly, day 12 fetal thymi were able to give rise to a significant number of NK1.1+ cells. When software gates were set to examine the CD4 vs CD8 expression of these NK1.1+ cells, we found that 52.1% of them were CD4+CD8−, 10.5% were CD4−CD8+, and 3.8% were CD4+CD8+ (Fig. 2D). Note that the thymocytes obtained from the FTOC established from day 14 fetal thymi also contained some NK1.1+ cells, although far less than those of day 12 FTOC (26.7 vs 9.2%, Fig. 2, A vs E). The CD4 vs CD8 expression of the NK1.1+ cells obtained from day 14 FTOC was also numerically different from those of day 12 FTOC, in that there was a higher percentage of CD8−CD4+ (Fig. 2, D vs H). There was even less CD4 or CD8 expression on day 16 and newborn NK1.1+ cells, although such cells were still detectable. Interestingly, the
NK1.1<sup>+</sup>CD<sub>4</sub><sup>-</sup>CD<sub>8</sub><sup>-</sup> cells (which are the cells demonstrable in the adult thymus) do not become the predominant NK1.1<sup>+</sup> cell until after birth (newborn FTOC, Fig. 2P). These data suggested that the first wave of thymic precursor cells may contain progenitors for NK1.1<sup>+</sup>CD<sub>4</sub><sup>-</sup>CD<sub>8</sub><sup>-</sup> cells, but that later waves do not. The amount of NK1.1<sup>+</sup> cells retrievable after 7 days of organ cultures decreased steadily from day 12 to 18 (Fig. 2 and data not shown), but then increased again in newborn thymi (Fig. 2, A, E, I, and M). Indeed, in data not shown (and Refs. 2-6), surface expression of CD<sub>3</sub>ε and TCR-αβ on the freshly examined NK1.1<sup>+</sup> thymocytes was not significant until after birth.

The data presented in Figure 2 suggested that the progenitors of NK1.1<sup>+</sup> thymocytes seen on day 15 to 16 of gestation were present in the thymi of day 12 fetuses. Several flow-cytometry experiments failed to show a significant number of NK1.1<sup>+</sup> cells in day 12 or 13 fetal thymi (data not shown). We sought to determine whether NK1.1 RNA is present in day 12 fetal thymi (as suggested by the FTOC experiment), and we also sought to determine the earliest...
The time point at which NK1.1 RNA can be demonstrated during gestation. Due to the paucity of obtainable cells, Northern blot analysis was not feasible. We, therefore, opted to perform RT-PCR analysis for NK1.1. For day 9 fetuses, the whole fetus was rendered into a single cell suspension from which RNA was obtained. RNA was also obtained from the supracardiac area of day 10 fetuses and from the thymic area of day 11 and 12 fetuses. RT-PCR analysis for NK1.1 and β-actin was performed, as detailed in Materials and Methods, and the product was analyzed on a 2.5% agarose gel.

We next examined the effect of cytokine supplementation of the FTOC on the maturation of CD4 and CD8 cells. As shown in Figure 6, A and E, FTOC cultured in medium alone for 7 days gave rise to the usual four subsets defined by CD4 vs CD8 expression; the majority of the CD8 cells were CD8αβ. Upon supplementation with IL-2, there were no CD4+ CD8+ nor CD4− CD8− cells; the CD4 CD8− cells were CD8αβ. Interestingly, FTOC supplemented with IL-7 were identical with those cultured alone, the only difference being the number of cells recovered with IL-7. IL-7-supplemented FTOC gave a fivefold higher cell yield. IL-12, on the other hand, seemed to skew the distribution of the thymocyte subsets toward CD8αβ (Fig. 6, D and H) cells, but with a decreased cell recovery. Similar findings were observed when the FTOC were examined after 14 days in culture (Fig. 7). When NK1.1+ cells were examined, we found that FTOC cultured with IL-7 had numbers and distribution of NK1.1+ CD3+ cells similar to FTOC cultured with medium alone. As shown above, FTOC cultured with IL-2 had an expanded NK1.1+ CD3− population. Interestingly, only the FTOC supplemented with IL-12 had an expanded NK1.1+ CD3− population. It is worth noting, however, that the number of cells recovered after 14 days of culture with IL-12 was much less than the number obtained from FTOC cultured with IL-7 or IL-2. In general, FTOC cultured with IL-12 for 14 days gave about 6 × 10^4 cells/fetal thymus, while FTOC cultured alone, with IL-2 or IL-7, gave about 7 × 10^4, 1.1 × 10^5, and 7 × 10^5 cells/fetal thymus, respectively.

**Lytic activity of fetal thymocytes**

We wondered whether the NK1.1+ cells in the IL-2-supplemented FTOC were lytically active. Ideally, we would have liked to sort these cells before use in the lytic assay; this was not feasible due to the limited number of cells. FTOC, established from day 14 fetal thymus, were cultured with medium alone or with IL-2 or IL-7 for 5, 7, or 10 days, after which single cell suspensions were prepared. Lytic activity was determined in a standard 4-h [51Cr] release assay against YAC-1 (the classical NK-sensitive target) and CL27A (NK-resistant, LAK sensitive (12, 17, 18)). As shown in Figure 8, FTOC cultured for 10 days with medium alone had no lytic activity, while IL-2-supplemented FTOC displayed a potent killing activity against YAC-1. Interestingly, CL27A targets were not killed, suggesting that the thymic killers are different from the classical LAK effectors (12, 17, 18). FTOC supplemented with IL-7 or IL-12 had no killing activity. Identical results were obtained when lytic activity was examined after 5 or 7 days of culture (data not shown).

**Discussion**

Thymic NK1.1+ cells are believed to develop later than conventional T cells in that they are not demonstrable before birth and do not reach adult levels until 5 to 10 wk of age (3–6). Most evidence in the literature suggests an extrathymic development of thymic NK1.1+ cells, although one report demonstrated their presence in FTOC established from day 15 fetuses (3–6, 10, 11). The presence of a cell with markers common to T cells and the classical CD3 NK cells prompted speculation about the developmental relationship of these two lymphocytes. Rodewald et al. (19) suggested that fetal thymocytes contain a progenitor that can develop into T cells or NK cells, depending on whether it matures in an intact thymic microenvironment. Sanchez et al. (20), using human fetal thymocytes cultured in murine FTOC, suggested the presence of a CD34bright progenitor capable of differentiating into either NK or T cells. Sanchez et al. (20) also found that CD34bright human
progenitors differentiated into NK cells, but not T cells, when cultured in suspension in the presence of IL-2. Our findings suggest that it is the cytokine exposure instead of, or in addition to, the microenvironment that may be pivotal in the development of NK cells.

Initially, we were interested in confirming that thymic NK1.1+ cells are present in day 14 to 15 thymocytes. Our approach was aimed at determining the earliest point at which thymic NK1.1+ cells can mature in FTOC. We thought that the data would be more meaningful if we first examined the presence of NK1.1+ cells in freshly obtained thymocytes. To our great surprise, we found a window of 12 to 24 h between day 15 and 16 of gestation, during which NK1.1+ cells are present in large numbers (range of 540%). At least 50% of these cells expressed B220 on their surface, a marker we have previously established to be expressed on IL-2- and IL-4-activated CD8+ T cells, CD3+NK cells, and CD3-NK cells (12, 17, 18). This peak of NK1.1+ cells was evident 12 to 24 h before the appearance of the CD4+CD8+ thymocytes, at which point the number of NK1.1+ cells dwindled to <5%. There could be several explanations for this finding. A trivial explanation is that the decrease of the NK1.1+ cells is dilutional. While we believe that there is a dilutional effect, we do not believe this to be the sole explanation since, in our hands, the number of recoverable thymocytes triples or, at most, quadruples between day 16 and 17, while the number of NK1.1+ cells decreases by 5- to 10-fold. Another trivial explanation is that the NK1.1+ cells leave the thymus to seed other organs. We have been unable to demonstrate such a peak in the liver, spleen, or intestines.

A third possibility is that the NK1.1+ cells develop into the CD4+CD8+ thymocytes. This possibility is strengthened by the fact that FTOC established from day 12 fetal thymus did indeed contain an NK1.1+CD4+CD8+ population. This population decreased with each day of gestation until it was hardly detectable by day 16. These findings suggest that the earliest thymic progenitors (first wave) may indeed contain a common progenitor for T and NK cells; later waves of progenitors appear to contain only T cell precursors. In support of this possibility is the observation of Rodewald et al. (21) that prothymocytes, which are T lineage committed, are present in the peripheral blood of athymic fetal mice. An unresolved question is our inability to demonstrate a triple-positive cell in freshly obtained fetal thymocytes. It is possible that such cells are subjected to negative selection in vivo (thymic NK cells are supposed to be positively selected by CD1, which is expressed on the CD4+CD8+ thymocytes (7)). A fourth possibility is that these cells die in the thymus or undergo apoptosis due to withdrawal of cytokines. Cytokine withdrawal is well known as a trigger of apoptosis (22-24). Indeed, it has been suggested that IL-2 and IL-4 may be present in the fetal thymus between day 14 and 16; these cytokines are not as abundant by day 17 of gestation (15). This possibility is strengthened by the fact that 50% of the fetal thymic NK1.1+ cells express B220 on their surface, suggesting that they were activated by cytokines. A more direct approach was to examine the fate of the various thymic subsets in FTOC supplemented with IL-2. As shown in Figure 5, day 14 FTOC supplemented with IL-2 retained their NK1.1+ cells (24%) when examined after 1 wk of culture, while the FTOC in culture medium alone had only 5.4% NK1.1+ cells. Interestingly, after 2 wk of IL-2 stimulation of the FTOC, the NK1.1+ cells overgrew most of the other subsets and accounted for 62.5% of all cells. The IL-2-supplemented FTOC did not contain CD4+CD8+ thymocytes, while the nonsupplemented FTOC had the usual distribution of thymocyte subsets. These findings suggest that IL-2 can support the growth of NK1.1+ thymocytes, but not the growth of CD4+CD8+ thymocytes.

An interesting observation in the cytokine-supplemented FTOC is that the CD8+ cells that developed in the IL-2-supplemented cultures were CD8α+β+, and were thus akin to the cells seen in intraepithelial lymphocytes (25-27). While IL-2 was capable of
FIGURE 5. NK1.1<sup>+</sup> cells in IL-2-supplemented FTOC. Intact thymic lobes were obtained from day 14 fetuses and were established in FTOC cultured in medium alone or in medium supplemented with 1000 U/ml of rHIL-2. After 7 or 14 days, the thymic lobes were retrieved and single cell suspensions were prepared and examined by flow cytometry.

FIGURE 6. One-week cultures of cytokine-supplemented FTOC. Day 14 fetal thymi were established in FTOC for 7 days, after which their surface expression of the indicated markers was examined by flow cytometry. The FTOC were cultured in medium alone or in medium supplemented with 1000 U/ml of rHIL-2, with 100 ng/ml of IL-7, or with 1 ng/ml of IL-12.

...skewing the thymocyte subset maturation, IL-7 was not; the cells recovered from IL-7-supplemented FTOC were similar to the cells recovered from FTOC cultured alone (except that cell yield was much higher in the case of IL-7). IL-12, on the other hand, skewed the thymocyte maturation toward CD8 single-positive cells, which were mostly CD8α<sup>+</sup>β<sup>+</sup>, although some CD8α<sup>-</sup>β<sup>-</sup> cells were evident at 2 wk of culture. Another intriguing finding of the NK1.1<sup>+</sup> cells demonstrated in the various FTOC is that they were mostly...
FIGURE 7. Two-week cultures of cytokine-supplemented FTOC. FTOC were established as in Figure 6, except that they were examined after 13 to 14 days in culture.

CD3ε−, with the exception of the IL-12-supplemented FTOC, wherein the majority of the cells were NK1.1+CD3+. It is possible that the CD3ε− cells express cytoplasmic CD3, as was shown for human fetal NK cells (28). We have attempted to examine the cytoplasmic CD3 expression of these cells, but were unable to obtain any meaningful data, since we obtained a very high background staining with all of the anti-CD3 mAb that we examined. Nevertheless, one can conclude from the data that IL-12 may play a significant role in the maturation, and perhaps surface marker expression, of CD3−NK1.1+ cells.

Although NK1.1+ cells are demonstrable in day 15 to 16 fetal thymi, it appears that the NK1.1+ gene is transcribed much earlier. As shown in Figure 3, utilizing RT-PCR, we could demonstrate transcription of NK1.1 as early as day 9 of gestation. Since the whole day 9 fetus was used, it is difficult to ascertain the organ in which NK1.1 is transcribed at that age. However, for day 10 fetuses, the supracardiac area was tested, suggesting that the thymus could be one organ in which NK1.1 was transcribed. For day 11, it was feasible to obtain the thymus and to demonstrate that NK1.1 is transcribed in that organ (Fig. 3). In data not shown, the fetal liver was obtained from day 11 fetuses, and we could demonstrate NK1.1 mRNA at that stage. It appears, therefore, that, at least on day 11 of gestation, the NK1.1 gene is transcribed simultaneously in the liver and the thymus. One issue raised by these findings is whether the NK1.1+ cells seen in the fetal thymus are precursors of the classical CD3NK cells or of the CD3−TNK cells. It is possible that early NK progenitors seed the thymus on day 9 or 10 of gestation and a subpopulation of these cells gives rise to the TNK cells; our data do not allow this conclusion at present.

Another interesting aspect of our data is that FTOC stimulated with IL-2 appear to favor the growth of NK1.1+ cells that are CD3− and are capable of killing the NK-susceptible target YAC-1, but not the NK-resistant target CL27A. These cells, therefore, appear similar to the classical NK cells. It is worth noting that Brooks et al. (29), using suspension cultures, also found that day 14 fetal thymocytes stimulated with IL-2 developed into cells that appeared to be indistinguishable from mature NK cells. More recently, Manoussaka et al. (30), utilizing day 14 fetal livers, found that high dose IL-2, plus IL-4 and PMA, allowed the development of NK-like cells (termed FL-A), while low dose IL-2 gave rise to NK1.1+2B4Ly49A+B20−noncytolytic cells (termed FL-B). The NK1.1+ cells seen in our IL-2-supplemented FTOC were 2B4+CD24−B220−Thy-1−CD4+CD2−Ly49A- (data not shown), and are thus more akin to the FL-A lines described by Manoussaka et al. (30). In view of these findings, it is
The data shown are LU per million cells; 1 LU was defined as the number of effectors needed to give a 30% specific lysis. possible that the CD3' NK1.1' cells seen represent a subset of classical NK cells, rather than the so-called TNK cells. We are in the process of addressing this question, and have thus labeled these cells as thymic NK1.1' cells with no assignment as to NK or TNK cells.

In summary, the data presented in this work demonstrate that NK1.1 is among the earliest of the transcribed lymphocytic genes in the mouse fetus, and that NK1.1' cells, or their progenitors, are among the earliest to seed the murine fetal thymus. It appears that the first wave of thymic progenitors contains a precursor that can give rise, in an intact thymic microenvironment, to an NK1.1' CD4' CD8' cell. Day 15 to 16 fetal thymus contain a significant number of NK1.1' cells that disappear by day 17; the role of this subset remains to be elucidated. It appears that IL-2, at least in FTOC, favors the growth of NK1.1' CD3' T cells (even in an intact thymic microenvironment), while IL-12 favors the growth of NK1.1' CD3' cells. These findings suggest that there may be numerous lymphocyte subsets in the fetal thymus that normally do not mature, but whose presence can be demonstrated upon expansion with certain cytokines.

References


