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NK T Cell Precursors Exhibit Differential Cytokine Regulation and Require Itk for Efficient Maturation

Paul Gadue*† and Paul L. Stein2†

NK T cells are a lymphocyte lineage that is selected by CD1d and is characterized by the ability to rapidly secrete large amounts of both IFN-γ and IL-4 after TCR stimulation. Using reactivity to CD1d tetramers to define presumptive NK T cells, several NK T cell progenitor populations were characterized based upon NK marker expression and CD4 vs CD8 expression. The earliest populations were found to be negative for NK markers and could proliferate to IL-7, while mature NK T cells did not. The NK1.1+ NK T cell progenitors were capable of up-regulating NK1.1 when transferred in vivo. Upon stimulation, the NK1.1+ populations secrete IL-4, but little IFN-γ. As the cells mature and up-regulate NK1.1, they acquire the ability to secrete IFN-γ. Finally, the Tec family tyrosine kinase Itk is necessary for optimal NK1.1 up-regulation and hence final maturation of NK T cells. The itk−/− mice also display a progressive decrease in NK T cells in older animals, suggesting a further role in peripheral maintenance. The Journal of Immunology, 2002, 169: 2397–2406.

Natural killer T cells are a unique lymphocyte lineage that express a TCR along with markers found on NK cells. The majority of these cells use a restricted TCR α-chain, Vα14α18, along with a β-chain skewed mainly to Vβ8.2 (1, 2). These cells are either CD4+ or negative for both CD4 and CD8 (double negative (DN)3) and express intermediate levels of TCR (3). They also are characterized by an activated phenotype, being CD69+, CD62Llow, and CD44high (3). NK T cells are restricted to the MHC-like molecule CD1d and can respond to glycolipids (4, 5). Consistent with this finding, CD1d tetramers loaded with the glycolipid, α-galactosylceramide (αGalCer), can bind to NK T cells (6, 7). One of the hallmarks of this cell lineage is its ability to quickly secrete large amounts of both Th1-type and Th2-type cytokines promptly after TCR ligation, suggesting an important immunoregulatory role (8, 9).

The developmental progression of the NK T cell lineage has several similarities and differences relative to conventional T cell development. Like conventional T cells, they are thymus derived, require the pre-TCR, and progress through a CD4+CD8+ (double positive (DP)) stage of development (3, 10–12). Unlike conventional T cells, they are selected by bone marrow-derived cells, most likely DP thymocytes (13, 14). They also show differential signal transduction requirements for their development. Dominant negative Ras and mitogen-activated protein/extracellular signal-related kinase kinase (MEK) transgenes disrupt conventional T cell development but have no effect on NK T cells, indicating that the Ras/Raf/MEK/mitogen-activated protein kinase cascade is not as important for NK T cell development as it is for conventional T cells (15). Conversely, the tyrosine kinase Fyn is dispensable for conventional T cell development but is required for proper NK T cell numbers (16, 17).

It has been shown that Src family members, such as Fyn, can activate other tyrosine kinases, which can in turn activate distinct biochemical pathways. One such kinase which is a substrate for Src kinases is the Tec family member Itk (18). Both itk and fyn mutant mice have a similar phenotype in regard to T cell development and antigenic responses. Both mice have near normal T cell numbers, but display decreased proliferation to TCR stimulation in thymocytes and to a lesser extent in peripheral T cells (19, 20). Moreover, itk/fyn double-mutant mice exhibit greatly attenuated TCR responses, suggesting that these two kinases may have partially overlapping functions (21). It is unknown whether Itk and Fyn share a common phenotype with regard to NK T cell ontogeny.

The studies presented here are aimed at obtaining a better understanding of NK T cell development and to further define the unique signal transduction requirements of this lineage. Several intermediate stages of NK T cell maturation are defined based upon NK marker expression on αGalCer-loaded CD1d tetramer-binding cells. The tetramer-positive cells found in neonatal mice do not express NK1.1. In contrast to mature NK T cells, this population also demonstrates an increased proliferative response to IL-7. Significant IFN-γ expression cannot be induced in this early population, but is gained in conjunction with NK1.1 up-regulation. Furthermore, this NK1.1-negative population can secrete greater levels of IL-4 than mature NK T cells. To begin defining mechanisms regulating maturation of NK T cells, itk-deficient mice were examined and found to have decreased numbers of tetramer-positive NK1.1+ cells. This suggests that Itk has an important role in the final differentiation events in NK T cell development.

Materials and Methods

Mice

C57BL/6 mice were bred on site. The itk−/− mice have been described previously (22) and were backcrossed to the C57BL/6 background five times. All mice used were at the ages indicated in individual experiments and maintained under American Association of Laboratory Animal Care and institutional guidelines.

*Graduate Group in Immunology and †Department of Dermatology, University of Pennsylvania, Philadelphia, PA 19104

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2 Address correspondence and reprint requests to Dr. Paul L. Stein, Department of Dermatology, University of Pennsylvania, 235A CRB, 415 Curie Boulevard, Philadelphia, PA 19104. E-mail address: steimp@mail.med.upenn.edu

3 Abbreviations used in this paper: DN, double negative; αGalCer, α-galactosylceramide; DP, double positive; MEK, mitogen-activated protein/extracellular signal-related kinase kinase; MEK1, mean fluorescence intensity; wt, wild type; SP, single positive.
Abs and reagents

The following Abs were used for flow cytometry: anti-mouse pan-NK cells-P (DX5), anti-mouse pan-NK cells-alkaline phosphatase (DX5), anti-NK1.1-PerCP-Cy5.5 (PK136), anti-NK1.1-alkaline phosphatase (PK136), anti-NK1.1-biotin (PK136), Fc Block (2.4G4), anti-IL-4-PE (BV42-4D11), anti-IL-4-alkaline phosphatase (BV42-4D11), anti-IFN-γ-FITC (XM12.1), anti-CD69-FITC (H1.2F3), anti-CD4-CyChrome (RM4-5), anti-CD4-PerCP-Cy5.5 (RM4-5), anti-CD8-allophycocyanin (53-6.7), anti-Ly-6C-FITC (AL-21), anti-CD44-FITC (IM7), CD122-biotin (TM-biotin), and streptavidin-FITC were purchased from BD Pharmingen (San Diego, CA). The following Abs were obtained from BD Pharmingen: anti-CD69-FITC (H1.2F3), anti-CD4-CyChrome (RM4-5), anti-CD4-PerCP-Cy5.5 (RM4-5), anti-CD8-allophycocyanin (53-6.7), anti-Ly-6C-FITC (AL-21), anti-CD44-FITC (IM7), CD122-biotin (TM-biotin), and streptavidin-FITC were purchased from BD Pharmingen (San Diego, CA). The following Abs were obtained from hybridoma supernatants: anti-MHC class II (MS/1415.2), anti-B220 (RA3-3A1/61), anti-CD8 (83-12-5; IgM), and anti-CD8 (2.4.3; IgG). GM-CSF was obtained from medium conditioned by the GM-CSF-secreting cell line J558L-GM-CSF. The αGalCer was obtained from Kirin Brewery (Gunma, Japan) and IL-7 was obtained from PeproTech (Rocky Hill, NJ). PE and TriColor-labeled CD1d tetramer loaded with αGalCer was a gift from Dr. M. Kronenberg (La Jolla Institute for Allergy and Immunology, La Jolla, CA) (8).

Flower cytometry

Single-cell suspensions of the thymus and spleen were obtained by disruption between two frosted microscope slides. Splenocytes were also depleted of RBCs by 0.14 M ammonium chloride treatment. All cells were treated with Fc Block for 15 min at 4°C. Intracellular staining was performed using the Cytotox/Cytoperm kit (BD Pharmingen) as per the manufacturer’s protocol. Three- and four-color immunofluorescence analysis was performed using a FACScan or FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences, San Jose, CA).

Cell purifications

NK T cells were enriched by either CD8 depletion or positive selection for tetramer-expressing cells. CD8 depletion was performed on thymocytes as follows. Cells were incubated with anti-CD8 (2.4.3) for 30 min, washed, then magnetically depleted using BioMag goat anti-rat IgG (Polysciences, Warrington, PA). This protocol depletes only CD8<sup>hi</sup> expressers. Selection for tetramer-positive cells was performed as follows. Cell suspensions were stained with PE-labeled αGalCer-loaded CD1d tetramer followed by anti-PE microbeads (Miltenyi Biotec, Auburn, CA). These cells were then positively selected using miniMACS MS separation columns as per the manufacturer’s protocol (Miltenyi Biotec). Dendritic cells were purified as described elsewhere (23). In brief, bone marrow was depleted of RBCs, then incubated with a mixture of anti-CD8 (83–12-5), B220, and class II Abs, followed by Low Tox-M rabbit complement (Accurate Chemical and Scientific, Westbury, NY). The remaining cells were cultured in 3% GM-CSF-conditioned medium at 1 × 10<sup>5</sup> cells/ml. After 6 days in culture, dendritic cells were prepared and gated as in Fig. 6 were analyzed for NK1.1, DX5, CD69, Ly-6C, CD122, or CD44. The percent positive for NK1.1, DX5, CD69 as well as high expressers for Ly-6C, CD44, and CD122 are shown for the indicated populations. The mean ± SD of three separate experiments are shown.

Results

Time-course expression of NK1.1 vs DX5 on NK T cell progenitors

It has been previously demonstrated that Var1418-expressing NK T cells are not present at significant numbers at birth, but start developing in neonates in the first few weeks of life (14, 24). NK T cells are also thought to develop in a stepwise fashion, first rearranging the NK T cell-specific TCR, then up-regulating NK markers such as NK1.1 (12, 25). To begin defining intermediate stages of NK T cell development, CD1d tetraments were used to identify presumptive NK T cell precursors. These progenitors were then analyzed to determine when NK T cells acquire their mature phenotype and function.

Thymocytes from C57BL/6 mice at different ages were examined to determine the precise timing of NK marker up-regulation. Both NK1.1, which recognizes NKR-P1C or CD161 (26), and DX5, recently discovered to recognize the α<sub>δ</sub> integrin or CD49b (27), were analyzed. The NK1.1 Ag is expressed on most mature NK T cells in C57BL/6 mice (7), while DX5 is present on most NK cells, but only a subset of mature NK T cells (28). However, another study indicated that CD1d tetramer-positive cells were DX5 negative (29). DX5 staining is lower on NK T cells than on NK cells and is difficult to detect when using the DX5 Ab conjugated to a weakly fluorescent fluorochrome such as FITC (P. Gaude and P. L. Stein, personal observations). The studies presented here use DX5-PE or DX5-allophycocyanin. Using these reagents, it is found that roughly half of mature peripheral tetramer-positive NK T cells are DX<sup>5+</sup> (Table I).

To define possible NK T cell progenitors, NK1.1 expression vs DX5 expression was examined on CD1d tetramer-positive thymocytes. Tetramer-positive thymocytes (Fig. 1A) from C57BL/6 mice ages 5 days, 2 wk, 4 wk, or 10 wk were examined for these two markers (Fig. 1B). Approximately one-third of the day 5 tetramer-positive cells express only the DX5 marker. Even fewer DX<sup>+</sup> cells are present in day 3 mice (data not shown), suggesting that acquisition of this marker may be developmentally regulated. Of particular note, in 5-day mice almost none of the tetramer-positive cells expressed NK1.1. By 2 wk of age, approximately one-third of the tetramer-positive cells express NK1.1, and nearly all of these cells are DX<sup>5–</sup> as well. At 4 wk, nearly half of the gated cells are positive for NK1.1 and a significant fraction emerge that do not express DX5. Thus, NK T cells from 4-wk-old mice have all four

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<th>Table I. NK T cell marker expression on DP&lt;sup&gt;low&lt;/sup&gt; and CD4&lt;sup&gt;hi&lt;/sup&gt;CD8&lt;sup&gt;−&lt;/sup&gt; populations&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fraction</th>
<th>% NK1.1&lt;sup&gt;+&lt;/sup&gt;</th>
<th>% DX5&lt;sup&gt;+&lt;/sup&gt;</th>
<th>% CD69&lt;sup&gt;+&lt;/sup&gt;</th>
<th>% Ly-6C&lt;sup&gt;hi&lt;/sup&gt;</th>
<th>% CD44&lt;sup&gt;hi&lt;/sup&gt;</th>
<th>% CD122&lt;sup&gt;hi&lt;/sup&gt;</th>
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<tr>
<td>CD4&lt;sup&gt;hi&lt;/sup&gt;CD8&lt;sup&gt;−&lt;/sup&gt;</td>
<td>8.4 ± 0.7</td>
<td>5.0 ± 1.6</td>
<td>5.0 ± 2.8</td>
<td>10.6 ± 1.7</td>
<td>31.2 ± 3.4</td>
<td>19.8 ± 5.1</td>
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<tr>
<td>DP&lt;sup&gt;low&lt;/sup&gt;</td>
<td>72.0 ± 3.3</td>
<td>46.4 ± 2.8</td>
<td>46.4 ± 4.8</td>
<td>34.4 ± 5.5</td>
<td>80.5 ± 2.9</td>
<td>73.9 ± 4.5</td>
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<tr>
<td>Spleenic NK T</td>
<td>65.3 ± 2.9</td>
<td>49.5 ± 3.2</td>
<td>68.7 ± 5.2</td>
<td>26.4 ± 2.2</td>
<td>92.9 ± 1.4</td>
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<sup>a</sup> CD4<sup>hi</sup>CD8<sup>−</sup> or DP<sup>low</sup> cells prepared and gated as in Fig. 6 were analyzed for NK1.1, DX5, CD69, Ly-6C, CD122, or CD44. The percent positive for NK1.1, DX5, and CD69 as well as high expressers for Ly-6C, CD44, and CD122 are shown for the indicated populations. The mean ± SD of three separate experiments are shown.
possible combinations of DX5 and NK1.1 expression. In fully mature 10-wk-old animals, NK1.1 is expressed on almost all tetramer-positive cells as previously reported (7). Furthermore, DX5 is expressed on less than half of these cells, which is consistent with DX5 only being present on a subpopulation of NK T cells (28). Peripheral NK T cells from 10-wk-old mice have a similar expression pattern as the 10-wk thymocytes, although the spleen may contain slightly fewer NK1.1+ cells (data not shown and Ref. 7). To control for nonspecific binding of tetramer, cells were stained with unloaded tetramer. This yielded <3% of the number of cells positive for αGalCer-loaded tetramer (data not shown). Moreover, the few cells positive for unloaded tetramer were negative for both NK1.1 and DX5 (data not shown), indicating that the DX5- and NK1.1-positive populations are αGalCer-reactive NK T cells.

It has been previously reported that embryonic day 13.5 fetal liver contains cells that express NK1.1 and the Vα14Jα18 TCR (30). To determine whether CD1d tetramers could detect this presumptive NK T cell population, fetal liver pooled from a litter of E13.5 embryos was examined. To enrich for TCR expressers, the subpopulations of NK T cells were analyzed in the various fractions (Fig. 2A). The most dramatic changes occur as the cells progress from fractions 2 to 3, which correlates with NK1.1 up-regulation. About 80% of the cells in fractions 1 and 2 express CD4, but expression is down-regulated in fractions 3 and 4 such that only ~50% are CD4+, a characteristic of mature NK T cells. The mean fluorescent intensity (MFI) respective (Fig. 2A). The first attributes analyzed on the presumed progenitors are TCR levels and cell size. It is thought that mature NK T cells have encountered an unknown endogenous Ag, hence they exhibit a characteristic activated phenotype that includes decreased levels of TCR expression (3). It would be predicted that before becoming activated, NK T cell progenitors may have higher levels of TCR similar to those observed on conventional T cells. Using tetramer to assess relative TCR density, fractions 3 and 4 are indicated. Fractions 1–4 display differences in cell size and TCR levels. Cell size was determined by forward scatter and TCR levels by tetramer staining. Expression of mature NK T cell markers is shown for fractions 1–4 by open histograms. Filled histograms are isotype controls. Percentage positive is indicated for CD4 and CD69. Percentage of high expressers is shown for Ly-6C and CD44 while MFI is shown for CD122. One of three independent experiments is shown.

**FIGURE 1.** Time course of NK1.1 and DX5 up-regulation in NK T cell progenitors. Thymocytes from 5-day-, 2-wk-, 4-wk-, and 10-wk-old C57BL/6 mice were enriched for NK T cells by CD8 depletion. Cells were then stained with αGalCer-loaded CD1d tetramers along with DX5 and NK1.1 Abs. A, Gate used for the dot plots shown in B. B, DX5 vs NK1.1 expression is shown for tetramer-positive cells. The percentage of cells in each quadrant is shown. The data shown are representative of four separate experiments.

**FIGURE 2.** Surface marker expression of different NK T cell progenitor fractions. Thymocytes from 4-wk-old C57BL/6 mice were enriched for NK T cells by CD8 depletion. All samples were stained with αGalCer-loaded CD1d tetramer. DX5 and NK1.1 Abs, along with either CD4, CD69, Ly-6C, CD44, or CD122. A, NK1.1 vs DX5 expression is shown for CD1d tetramer-positive cells. Fractions 1–4 are indicated. B, Fractions 1–4 display differences in cell size and TCR levels. Cell size was determined by forward scatter and TCR levels by tetramer staining. C, Expression of mature NK T cell markers is shown for fractions 1–4 by open histograms. Filled histograms are isotype controls. Percentage positive is indicated for CD4 and CD69. Percentage of high expressers is shown for Ly-6C and CD44 while MFI is shown for CD122. One of three independent experiments is shown.

Surface phenotype characterization of NK T cell progenitor subpopulations

Markers characteristic of mature NK T cells were examined on the various tetramer-positive populations described in Fig. 1B to determine whether they exhibit differences in expression. The populations: DX5+NK1.1+, DX5+NK1.1−, DX5−NK1.1+, and DX5−NK1.1− will now be referred to as fractions 1, 2, 3, and 4, respectively (Fig. 2A). The first attributes analyzed on the presumed progenitors are TCR levels and cell size. It is thought that mature NK T cells have encountered an unknown endogenous Ag, hence they exhibit a characteristic activated phenotype that includes decreased levels of TCR expression (3). It would be predicted that before becoming activated, NK T cell progenitors may have higher levels of TCR similar to those observed on conventional T cells. Using tetramer to assess relative TCR density, fractions 1 and 2 were found to have higher levels of tetramer staining than fractions 3 and 4 (Fig. 2B). This is consistent with these cells being earlier progenitors that have yet to become activated by Ag and down-regulate TCR levels. Fractions 3 and 4 have similar levels of tetramer expression as mature NK T cells (data not shown). Fractions 1 and 2 are larger, consistent with a cycling blast-like state. In contrast, the cells in fractions 3 and 4 are smaller (Fig. 2B) and are similar in size to mature NK T cells from either the thymus or periphery of 10-wk-old mice (data not shown), suggesting that these fractions represent more mature NK T cells.

Mature NK T cells have a characteristic expression pattern of cell surface markers that distinguish them from naive conventional T cells. NK T cells are either CD4+ or DN with regard to coreceptor expression. In addition, they are CD69+, CD44high, CD122+, and a proportion are Ly-6Chigh (3). All of these markers were analyzed in the various fractions (Fig. 2C). The most dramatic changes occur as the cells progress from fractions 2 to 3, which correlates with NK1.1 up-regulation. About 80% of the cells in fractions 1 and 2 express CD4, but expression is down-regulated in fractions 3 and 4 such that the mean fluorescent intensity (MFI)
of CD4 on tetramer-binding cells is also decreased in fractions 3 and 4, relative to 1 and 2 (data not shown). In fractions 1 and 2, <10% of the cells are CD69+, but this increases to ~70% in fractions 3 and 4. The remaining markers examined show a more gradual change in expression in fractions 1 through 3, consistent with these three fractions representing progressive maturational steps. The expression level of Ly-6C and CD44 is relatively low in fractions 1 and 2. As the cells move into fractions 3 and 4, expression of these two markers increases considerably, so that nearly all of the cells are CD44^high and half are Ly-6C^high. All fractions are positive for CD122, but the MFI increases from 6.5 to 10 to 13.5 in fractions 1 through 3, respectively. All of these data are suggestive of a stepwise maturation from fractions 1 to 2 to 3.

For all surface markers examined, fractions 3 and 4 express similar levels as mature NK T cells (data not shown and Table I).

**IL-7 responsiveness of NK T cell progenitors**

Mature NK T cells do not proliferate to a significant extent in response to IL-7, but use it as a survival factor (Ref. 31 and data not shown). IL-7 can enrich for NK T cells in thymocyte cultures, suggesting a possible proliferative response by NK T cell progenitors present in the thymus (32). Because of the blast-like character of fractions 1 and 2 (Fig. 2B), it could be predicted that these earlier progenitors may respond to IL-7 by proliferation while later ones would not. Thymocytes from mature mice were labeled with CFSE and cultured with IL-7 for 4 days. Live cells from this culture were isolated and the CFSE content was examined in the four fractions of tetramer-positive cells (Fig. 3A). As shown in Fig. 3B, fractions 1 and 2 both divide to a significant extent as seen by the relative decrease in CFSE content per cell. Fraction 3, in contrast, exhibits only a modest amount of cell division while virtually no proliferation occurs in fraction 4. When cultured in the absence of IL-7, few cells were obtained in any fraction (data not shown), indicating that this cytokine can provide survival as well as cell growth signals. Thus, consistent with the larger cell size of fractions 1 and 2, a characteristic of dividing cells, these populations are capable of responding to IL-7 by proliferation. In contrast, the smaller sized fractions 3 and 4 appear to use IL-7 mainly as a survival factor at the concentrations used in this study. These data give further support to the idea that fractions 1 and 2 represent a more immature population of NK T cells.

It is possible that IL-7 may promote differentiation of the fraction 1 cells. To test this hypothesis, thymocytes from 4-wk-old mice were cultured with IL-7 to expand progenitors and enrich for NK T cells. After 4 days with IL-7, live cells were isolated, then depleted of cells expressing DX5 and NK1.1 (fractions 2, 3, and 4). The fraction 1-enriched cells were then cultured with or without IL-7 to determine its effect on maturation. In the absence of IL-7, only 3.4% of the cells expressed DX5. When IL-7 was included during the culture, ~25% of the population was DX5+ (Fig. 3C). These culture conditions did not allow for significant NK1.1 up-regulation (data not shown). Thus, not only does IL-7 induce proliferation of fractions 1 and 2, it also has the ability to promote up-regulation of DX5.

**NK1.1+ precursors develop into NK1.1+ cells after in vivo transfer**

Although the experiment presented above demonstrates that fraction 1 cells can develop into fraction 2 cells, it is still unknown whether fraction 1 and 2 cells (NK1.1+) can develop into fraction 3 and 4 cells (NK1.1++). To address this issue, IL-7-expanded NK T cell progenitors were depleted of NK1.1+ expressing cells (Fig. 4A), then labeled with CFSE and transferred into C57BL/6 mice via i.v. injection. CFSE-labeled tetramer-positive cells from both the spleen and liver show little NK1.1 expression 1 day after transfer (Fig. 4B). However, after 6 days, 38% of the liver and 33% of splenic tetramer+ CFSE+ cells express NK1.1. In addition, cell division does not appear to be required for this differentiation step as indicated by the CFSE content of the NK1.1+ cells (Fig. 4B). The lack of cell division also rules out the possibility of selective outgrowth from a small number of NK1.1+ contaminants in the transferred cell preparation. To confirm that more immature thymic progenitors (tetramer negative) present in the cell preparations were not directly developing into tetramer-positive NK1.1+ cells, the cultures were depleted of tetramer-expressing cells before transfer into mice. The number of tetramer-positive CFSE+ cells found in mice receiving tetramer-depleted cells was reduced >50-fold relative to animals given nondepleted cells (data not shown). This indicates that the tetramer-positive NK1.1+ cells are derived from the tetramer-positive NK1.1+ cells transferred. These data confirm the hypothesis that NK1.1- cells can develop into NK1.1+ cells.

**Induction of cytokines in NK T cell progenitors**

Data presented here suggest that NK T cells undergo a complex maturation process involving the sequential up-regulation of the DX5 and NK1.1 markers. NK T cells have the ability to rapidly
secrete large amounts of both Th1 and Th2 cytokines, such as IFN-γ and IL-4, respectively (8, 9). To determine whether acquisition of cytokine synthesis was developmentally regulated, the fractions described above were examined for the ability to secrete IFN-γ and IL-4 upon stimulation (Fig. 5A). NK T cell-enriched thymocytes from 4-wk-old mice were stimulated in vitro with PMA and ionomycin. As the NK T cell precursors transit from fractions 1 to 3, there is a progressive increase in the number of cells that are capable of secreting IFN-γ. Only 33% of fraction 1 cells produce IFN-γ and approximately half of fraction 2 cells are able to secrete this cytokine. In contrast, >90% of fractions 3 and 4 are capable of IFN-γ synthesis. Based on the MFI, there is no difference in the amount of IFN-γ made on a per cell basis in any of the fractions, even though fewer fraction 1 and 2 cells synthesize it. These data demonstrate that IFN-γ production is gained late in NK T cell ontogeny in conjunction with NK1.1 up-regulation.

IL-4 production in the various fractions appears to be regulated differently than IFN-γ. Fractions 1 and 2 contain a slightly higher percentage of IL-4-positive cells (~70%) than fractions 3 and 4 (~50%; Fig. 5A). However, fraction 1 and 2 cells secrete considerably more cytokine than fractions 3 and 4 on a per cell basis. Of the IL-4-positive cells, fractions 1 and 2 have a MFI of ~115 while the MFI in fractions 3 and 4 is reduced to only ~30. These data suggest that the ability to produce IL-4 is gained earlier than IFN-γ and before NK1.1 up-regulation. In addition, as the cells acquire the ability to make IFN-γ, the amount of IL-4 made decreases ~4-fold.

The experiments described above use PMA plus ionomycin treatment to induce cytokine production. To confirm these results with a more physiologic stimulus, NK T cell progenitors were stimulated with dendritic cells pulsed with the NK T cell ligand GalCer. Thymocytes from 4-wk-old mice were enriched for NK T cells and cultured overnight with IL-7 to increase IL-4 production (33, 34). Parallel experiments were also conducted in the absence of IL-7 with similar results, although the magnitude of the IL-4 response was much lower (data not shown). Both IFN-γ and IL-4 production were examined in fractions 1 and 2 (NK1.1⁺) and fractions 3 and 4 (NK1.1⁻) (Fig. 5B). As observed with the PMA and ionomycin stimulus, considerably more cells make IFN-γ in fractions 1 and 2 (61%) than in fractions 1 and 2 (22%). Like the chemically stimulated cells, the numbers of cells making IL-4 is slightly higher in fractions 1 and 2 (50%) vs fractions 3 and 4 (39%), and the NK1.1-negative fractions also secrete more IL-4 per cell than the NK1.1-positive fractions. These data confirm the idea that NK T cells first gain the ability to synthesize IL-4 and later gain the ability to make IFN-γ in conjunction with NK1.1 up-regulation.

**DPlow cells appear late in NK T cell ontogeny**

It has been suggested that NK T cells go through a CD4 and CD8 DPlow stage during their development (12). This population was present only in the thymus of young mice and was nearly undetectable by 6 wk of age (Fig. 6A). To determine whether this population consists of immature or mature NK T cells, the expression of NK1.1 on NK T cell-enriched thymocytes from 2-wk-old mice was examined. Tetramer-positive cells were gated on NK1.1⁺ or
NK1.1<sup>+</sup> and CD4 vs CD8 expression was determined (Fig. 6B). Approximately half of the NK1.1<sup>+</sup> cells from 2-wk-old mice were DP<sub>low</sub>, while only ~10% were CD4<sup>high</sup>CD8<sup>−</sup>. A reciprocal pattern was found in the NK1.1<sup>−</sup> pool. Nearly half the cells were CD4<sup>high</sup>CD8<sup>−</sup>, while only 10% were DP<sub>low</sub>. In addition, CD4<sup>high</sup>CD8<sup>−</sup> cells are larger than DP<sub>low</sub> cells, in a manner reminiscent of the differences between fractions 1/2 and 3/4 (Figs. 6C and 2B). These results suggest that at 2 wk of age DP<sub>low</sub> cells consist of more mature NK1.1<sup>+</sup> cells, while CD4<sup>high</sup>CD8<sup>−</sup> cells exhibit a more immature NK1.1<sup>−</sup> phenotype.

To confirm whether CD4<sup>high</sup>CD8<sup>−</sup> cells represent immature NK T cells and DP<sub>low</sub> cells represent a late stage in NK T cell ontogeny, other developmentally regulated surface markers were examined on CD1d tetramer-positive thymocytes. Like fraction 1 cells, most CD4<sup>high</sup>CD8<sup>−</sup> cells do not express the NK markers NK1.1, DX5, nor do they express other markers associated with mature NK T cells such as CD69 and Ly-6C (Table I). They also have low levels of CD122 and fewer CD44<sup>high</sup> cells. All of these data suggest that this population falls within fraction 1. In contrast, the DP<sub>low</sub> cells have a phenotype similar to more mature NK T cells (Table I). They express both NK cell markers DX5 and NK1.1, as well as CD69<sup>+</sup> and CD44<sup>high</sup>, and having cells which are CD69<sup>+</sup> and Ly-6C<sup>high</sup>. All of these data suggest that DP<sub>low</sub> cells may be more representative of a late stage in NK T cell development, such as fraction 3.

The results described above are compatible with a relatively complex developmental pathway. The early NK T cells may start as CD4<sup>high</sup>CD8<sup>−</sup> cells that are negative for most of the mature NK T cell markers (fraction 1). These cells may up-regulate CD8 and down-regulate CD4, leading to DP<sub>low</sub> cells. Consistent with this idea, an intermediate population of CD4<sup>high</sup>CD8<sup>−</sup> can be seen in the NK1.1<sup>+</sup> fraction of 2-wk mice (Fig. 6B). At this stage surface TCR density would decrease and mature NK T cell markers would begin to express. The final stage would then involve DP<sub>low</sub> developing into either CD4<sup>+</sup> or DN cells.

**FIGURE 6.** DP<sub>low</sub> NK T cells represent a late stage in NK T cell development. Thymocytes and splenocytes from 2- or 6-wk-old mice were enriched for NK T cells by staining with PE-labeled CD1d tetramer and positive selection of tetramer-positive cells using anti-PE magnetic beads. These cells were further stained for CD4 and CD8. A, CD4 vs CD8 expression is shown for cells gated as indicated. Percentages of tetramer-positive cells that are CD4<sup>high</sup>CD8<sup>−</sup> and DP<sub>low</sub> are indicated. B, Cells were stained as in A with the addition of NK1.1 staining. Percentages of CD4<sup>high</sup>CD8<sup>−</sup> and DP<sub>low</sub> populations on NK1.1<sup>−</sup> or NK1.1<sup>+</sup> gated cells are indicated. These gates are also shown for tetramer-negative conventional T cells as a comparison (right-most plot). Note the typical SP and DP cell populations. C, Cells from 2-wk-old wt thymus were stained and gated for DP<sub>low</sub> and CD4<sup>high</sup>CD8<sup>−</sup> cells as in A. Cell size, determined by forward scatter, is shown. The shaded histogram represents CD4<sup>high</sup>CD8<sup>−</sup> cells while the unshaded histogram represents DP<sub>low</sub> cells. One of three representative experiments is presented. lo, Low; hi, high.

NK T cell precursors in itk<sup>−/−</sup> mice contain a partial block in NK T cell development at fraction 2

NK T cells differ from conventional T cells in terms of the signal transduction network required for their development. These two cell types show differential requirements for the Fyn tyrosine kinase and the Ras/MEK pathway (15–17). Therefore, it might be anticipated that downstream targets of these molecules should also affect NK T cell development.

Itk is a member of the Tec family of tyrosine kinases. Upon phosphorylation by Src family members such as Fyn, Itk becomes activated and can induce distinct signal transduction pathways (18). To determine whether Itk may provide signals regulating NK T cell development, the populations discussed above were examined in itk<sup>−/−</sup> mutant mice.

Tetramer-positive cells from 2-wk-old wild-type (wt) and itk<sup>−/−</sup> mice were examined for DX5 vs NK1.1 expression and CD4 vs CD8 expression (Fig. 7, A and B). Mice deficient in itk have twice the percentage of fraction 2 cells as wt, with a concomitant reduction in fraction 3 cells, suggesting that itk mutant mice may have a partial block in development at fraction 2. If this were the case, the DP<sub>low</sub> cells may be decreased since this population appears to fall into fraction 3. As predicted, the DP<sub>low</sub> cells may be decreased since this population appears to fall into fraction 3. As predicted, the itk mutants have fewer DP<sub>low</sub> cells, with the wt containing 29% and the itk<sup>−/−</sup> mice having only 3.4% (Fig. 7B). In contrast, CD4<sup>high</sup>CD8<sup>−</sup> cells increase from 33% in the wt to 75% in the itk mutant. Examining splenocytes from 20-wk-old itk<sup>−/−</sup> mice reveal a consistent decrease in the number of NK1.1<sup>+</sup> tetramer<sup>−</sup> cells (Fig. 7C). In conjunction with this lack of NK1.1 up-regulation, the itk mutant NK T cells fail to efficiently express CD69. Both of these markers are expressed at roughly half the levels seen in wt tetramer<sup>+</sup> cells (Fig. 7C). These data suggest that itk<sup>−/−</sup> mice contain a partial block in NK T cell maturation from CD4<sup>high</sup>CD8<sup>−</sup> to DP<sub>low</sub> cells or from fraction 2 to fraction 3.

The amount of total NK T cells, as defined by tetramer-positive cells, was examined in itk<sup>−/−</sup> mice to determine how the partial
developmental block may affect NK T cell numbers. Cellularity of both the thymus and spleen in Itk−/− mice was similar to wt (Ref. 20 and data not shown). The percentage of tetramer-positive cells was increased ~2-fold in the thymus of 2-wk Itk−/− mice (Fig. 7D). However, by 6–8 wk the Itk−/− thymus contains similar numbers as wt. Over time there is a progressive loss of NK T cells in the Itk mutant, so that by 20 wk the Itk−/− mice have approximately a 3-fold decrease in the numbers of tetramer-positive cells compared to wt (Fig. 7D). The reduction in NK T cell numbers is accelerated in the periphery. The decrease is clearly evident in spleens from 6- to 8-wk mice and the total numbers continue to drop as the mice age. These data suggest that Itk may be important in peripheral upkeep or homeostatic proliferation.

Discussion

NK T cell progenitor populations defined by surface marker expression

NK T cell ontogeny is still poorly understood in comparison to conventional T cell development. The advent of tetramer technology now allows for the examination of cells solely on the basis of the NK TCR expression instead of following the most mature cells that express the TCR along with NK cell markers. Thus, tetramer allows the characterization of the intermediate stages of NK T cell development. Already, a previous study using αGalCer-loaded CD1d tetramers has revealed several important pieces of information about their developmental pathway. Young mice contain a large proportion of tetramer-positive cells that lack NK1.1 and activation markers, as well as cells that display a DP high phenotype (12). In this study, tetramer-positive cells have been further characterized in young mice, demonstrating that the cells can be further divided into discrete subsets that have fundamentally different proliferative capacities to IL-7 and cytokine expression profiles.

The results reported here suggest a complex developmental pathway for NK T cells (summarized in Fig. 8). The first tetramer-positive cells detected in young 5-day mice are a mix of DX5+ and DX5− cells (fractions 1 and 2), but are negative for NK1.1 as well as most other mature NK T cell markers (Figs. 1 and 2). These cells are also larger and have higher levels of TCR relative to mature NK T cells. By 2 wk of age a new population emerges which express both DX5 and NK1.1 (fraction 3). These cells have characteristics of mature NK T cells, such as intermediate TCR levels and expression of activation markers. The cells from 2-wk-old thymus are also enriched for DP high expression of CD4 and CD8. The fraction 3 (NK1.1+) cells from older mice contain very few DP high cells, suggesting that the DP high population is transient and probably is indicative of newly matured NK T cells. This is consistent with 5-bromo-2′-deoxyuridine-labeling studies which

FIGURE 7. Itk−/− mice contain decreased amount of DP high and fraction 3 NK T cells. Thymocytes from 2-wk-old wt and itk−/− mice were enriched for NK T cells as in Fig. 6. A. The cells were further stained with NK1.1 and DX5. Expression of these two markers on tetramer-positive cells is shown. Percentage of cells in each quadrant is indicated. Note that the itk mutant has an increased percentage of fraction 2 cells. B. Expression of CD4 and CD8 on tetramer-positive cells. Gates for CD4 high/CD8 low and DP high populations are shown and the percentage of cells in these gates is indicated. One of three independent experiments is shown. C. Splenocytes from 20-wk-old wt and itk−/− mice were stained with CD1d tetramer, CD69, and NK1.1. Histograms of NK1.1 or CD69 expression are shown gated on tetramer-positive cells. The dotted lines represent wt mice and the solid line represents itk mutant mice. The percentage of positive cells is indicated. D. Thymocytes and splenocytes from 2-, 6- to 8-, and 20-wk-old mice were stained with CD1d tetramer. The percentage of CD1d tetramer-positive cells per organ was calculated and shown for both wt and itk−/− mice. Organ cellularity was similar between the wt and itk mutant mice; therefore, percentages are directly proportional to absolute numbers. Note the age-dependent decrease in tetramer-positive cells in itk−/− mice. Data shown are representative from at least three separate experiments. Two-week: wt, n = 4; itk−/−, n = 4; 6- to 8-wk: wt, n = 4, itk−/−, n = 4; 20-wk: wt, n = 5, itk−/−, n = 6. Spl, Spleen; Thy, thymus.

FIGURE 8. Functional and phenotypic changes during NK T cell maturation. The proposed stages of NK T cell maturation are shown in the center portion of this figure. The data presented in this report are summarized for both cytokine production and surface marker expression. hi, High; int, intermediate.
demonstrated that NK T cell production occurs in the thymus during the first few weeks of life, with little labeling occurring in older mice (14).

During the review of this paper, two other works were published with results relevant to this study (35, 36). These studies, as well as those presented here, illustrate that the NK1.1+ fraction can develop into the NK1.1+ population. In addition, all three studies demonstrate that thymic tetramer-positive cells have differential cytokine production profiles based upon NK1.1 expression. The studies presented here also extend these findings, analyzing IL-7 responsiveness and demonstrating that the final maturation and NK1.1 up-regulation does not require cell division. Data presented here also highlight the importance of Itk in mediating up-regulation of NK1.1 and activation markers. These results provide a possible role for Itk-based signals as a mechanism for the final maturation event during NK T cell ontogeny.

A previously published work demonstrated the existence of a transient population of tetramer+ DP-low cells (12). These cells have been further analyzed in this study and were shown to exhibit characteristics of mature NK T cells, including relatively small size. However, Pellicci et al. (36) failed to detect these DP-low cells. The few DP cells identified were described as large and consisted of doublets. It was suggested that MACS enrichment of tetramer-positive cells may have led to the discovery of DP-low cells. However, DP-low cells can be detected in bulk thymocytes without MACS enrichment (data not shown). Alternatively, since this population expresses very low levels of both CD4 and CD8, it can be difficult to distinguish with certain fluorochrome/Ab combinations. Although Pellicci et al. (36) propose that the first NK1.1-positive cells appearing in young mice tend to be DN, the studies presented here would suggest that this DN population is actually DP-low. It is possible that the DP-low stage represents cells that are in the process of becoming either CD4 or DN. Further experimentation is needed to confirm this hypothesis.

**IL-7 responsiveness in NK T cell progenitors**

IL-7 has been shown to be an important growth factor for immature T cell progenitors, but has a decreased ability to induce proliferation of mature T cells. The fact that fractions 1 and 2 proliferated to a greater extent than the NK1.1+ fractions (3 and 4) supports the notion that fractions 1 and 2 may be progenitors of fractions 3 and 4 (Fig. 3B). These results may have parallels with the pattern of IL-7 responsiveness found during conventional T cell ontogeny. Early DN thymocytes divide upon IL-7 addition, while more mature DP thymocytes fail to respond to this cytokine (37, 38). IL-7-induced proliferation is regained in the single-positive (SP) thymocyte stage, but is decreased again in peripheral mature T cells (38). Although it has been difficult to identify a definitive DP-high stage of NK T cell development by staining with CD1d tetramer, NK T cells can develop from a DP+ TCRαβ+ thymocyte following intrathymic injection (12). Thus, all of the fractions described here develop after this DP stage since they recognize tetramer and have therefore rearranged their α locus. Based on IL-7 responsiveness, fractions 1 and 2 may be similar to SP thymocytes while fractions 3 and 4 would be more similar to mature peripheral T cells.

**Developmental regulation of cytokine production**

One of the hallmarks of NK T cells is their ability to secrete large amounts of both IL-4 and IFN-γ upon TCR stimulation. The ability to synthesize these cytokines is developmentally regulated. Fractions 1 and 2 secrete mainly IL-4 at high levels, but virtually no IFN-γ. Moreover, the amount of IL-4 is actually higher than what is made by mature NK T cells (Fig. 5). This suggests that the ability to make IL-4 is gained first in development, followed by IFN-γ. Human NK T cells may undergo a similar developmental progression. NK T cells isolated from human cord blood can be skewed to a Th2 or Th1/Th2 mix phenotype by culturing with different subsets of dendritic cells (39). In contrast, NK T cells cultured from mature blood do not develop a strong Th2 bias. This fits well with the model presented here if cord blood contains more immature Th2 like NK T cells which can be expanded with the proper conditions. Mature human NK T cells can be split into two functionally distinct fractions based on CD4 expression as well. The CD4+ population can secrete both Th1 and Th2 cytokines while the CD4− population secretes mainly Th1 cytokines (40, 41). This Th1 population may represent a further stage of differentiation past the Th1/Th2 mixed NK T cell.

The dynamic changes in cytokine secretion profiles during NK T cell ontogeny shares some striking parallels with those observed during NK development. Early human NK cell progenitors make solely Th2 cytokines, such as IL-13, and then differentiate to a cell that can make both Th2 and Th1 cytokines and start expressing other NK markers such as CD56. As the NK cell undergoes final maturation, it develops into cells which only make IFN-γ, but no IL-13 (42). Similarly, the data presented here suggest that during mouse NK T cell development, mature NK cell markers are up-regulated in conjunction with the ability to synthesize IFN-γ.

In contrast to NK and NK T cells, conventional T cells are thought to acquire the ability to produce IL-4 or IFN-γ by a different mechanism. The naive population first produces mainly IL-2, then begins to make either Th1 cytokines, such as IFN-γ, or is induced to differentiate into a population that makes Th2 cytokines, such as IL-4 (43). Further study of NK T cell maturation may provide important insights for understanding how NK T cells and conventional T cells acquire their distinct cytokine expression patterns, and hence effector functions.

Understanding NK T cell cytokine regulation may provide insight into certain human disease states. Studies with identical twins discordant for juvenile diabetes demonstrated skewed cytokine production by NK T cells, with the diabetic twin’s NK T cells making IFN-γ, with no IL-4 (44). In contrast, NK T cells from prostate cancer patients show diminished IFN-γ production, but still make IL-4 (45). These studies highlight the importance that differential cytokine production by NK T cells may play in immune regulation.

**The role of Itk in NK T cell ontogeny**

The data presented here suggests that Itk is required at two distinct points in NK T cell homeostasis. The increase in fraction 2 (NK1.1−) cells at the expense of fraction 3 (NK1.1+) in the mutant suggests that Itk may be important in the final steps of NK T cell maturation. This would include NK1.1 up-regulation in conjunction with other activation markers that are found on mature NK T cells. Fraction 2 may be a stage where significant cell expansion occurs during NK T cell development, since they are blast-like and divide in response to IL-7. If the precursors cannot progress past this stage, the absolute number of tetramer-positive cells may increase, as seen in young itk mutant mice (Fig. 7D). Therefore, the itk mutation may interfere with the transition from proliferating to quiescent populations, leading to an increase in NK T cell precursors.

Itk may have a separate role in peripheral maintenance of NK T cells, since the itk mutant mice undergo a progressive loss of NK T cells in older mice. It is possible that this defect is masked in younger mice due to increased proliferation of immature progenitors, as 5-bromo-2′-deoxyuridine-labeling studies show the majority of thymic NK T cell expansion occurs in the first few weeks
of life (14, 24). One potential mechanism for the loss of NK T cells in older animals would be that expression of antiapoptotic genes may be affected. However, Bcl-2 and Bcl-x protein levels were found to be similar in wt and itk−/− NK T cells (data not shown), indicating that other deficiencies may be present leading to the decreased NK T cell numbers in the itk mutants.

The Src family tyrosine kinase Fyn is necessary for maintaining proper numbers of NK T cells in both the thymus and periphery while NK and conventional T cell development are largely unaffected by the fyn mutation (16, 19, 46). The possibility that the decreased NK T cell numbers in the fyn-deficient background is due to faulty Itk activation is probably not the case. All of the fractions described here are reduced in the itk mutant mice, suggesting a block before fraction 1 (data not shown). In contrast, the itk mutants may have a partial defect occurring at the junction of fractions 2 and 3.

The phenotype of the itk−/− mice reveals some additional insights into NK T cell ontogeny. The DBlow stage of NK T cell development may not be required since this population is virtually absent in itk mutant mice but mature NK1.1+ cells still develop, although at a decreased frequency. The NK1.1+ NK T cells in itk−/− mice are also functionally mature because they secrete IFN-γ when stimulated by PMA and ionomycin (data not shown). The fact that a more complete block in NK T cell development does not occur may be due to compensation by other family members. Itk is a member of a larger gene family, that includes Rlk and Tec, both of which are expressed in T and NK cells (47). It has insights into NK T cell ontogeny. The DP low stage of NK T cell development is selectively impaired in Fyn-deficient mice. J. Immunol. 166:2412.

References

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References


