Lineage Relationships and Differentiation of Natural Killer (NK) T Cells: Intrathymic Selection and Interleukin (IL)-4 Production in the Absence of NKR-P1 and Ly49 Molecules

By Olivier Lantz,* Lama I. Sharara,† Florence Tilloy,,* Åsa Andersson,‡ and James P. DiSanto*‡

Summary

In this report, we have assessed the lineage relationships and cytokine dependency of natural killer (NK) T cells compared with mainstream TCR-αβ T cells and NK cells. For this purpose, we studied common γ chain (γc)-deficient mice, which demonstrate a selective defect in CD3ε-NK cell development relative to conventional TCR-αβ T cells. NK thymocytes differentiate in γc− mice as shown by the normal percentage of TCR Vβ8+ CD4−CD8− cells and the normal quantity of thymic Vα14-Jα281 mRNA that characterize the NK T repertoire. However, γc-deficient NK thymocytes fail to coexpress the NK-associated markers NKR-P1 or Ly49, yet retain characteristic expression of the cytokine receptors interleukin (IL)-7Rα and IL-2Rβ. Despite these phenotypic abnormalities, γc− NK thymocytes could produce normal amounts of IL-4. These results define a maturational progression of NK thymocyte differentiation where intrathymic selection and IL-4-producing capacity can be clearly dissociated from the acquisition of the NK phenotype. Moreover, these data suggest a closer ontogenetic relationship of NK T cells to TCR-αβ T cells than to NK cells with respect to cytokine dependency. We also failed to detect peripheral NK T cells in these mice, demonstrating that γc-dependent interactions are required for export and/or survival of NK T cells from the thymus. These results suggest a stepwise pattern of differentiation for thymically derived NK T cells primary selection via their invariant TCR to confer the IL-4-producing phenotype, followed by acquisition of NK-associated markers and maturation/export to the periphery.

NK T cells are a specialized subset of T cells that share surface markers with the NK cells and have unique properties with respect to their TCR diversity and specificity, as well as their ultimate biological functions (1–3). NK T cells comprise both CD4− and CD8− (double negative, DN) and CD4+ TCR-αβ bearing T cells (4, 5), which coexpress a cluster of NK cell markers, including receptors of the C-lectin Ly49 and NKR-P1 (including the NK1.1 antigen) families (1–3, 6, 7). NK T cells express high levels of the IL-2Rβ molecule, a shared cytokine receptor chain used by IL-2 and IL-15, which is also found on NK cells and TCR-γδ T cells, but at low levels on conventional TCR-αβ T cells (8). Moreover, although the level of TCR expression on conventional T cells is high, NK T cells express intermediate TCR levels (TCR-αβint). The TCR-αβ repertoire of NK T cells is markedly restricted; TCR-β chain usage includes Vβ8, Vβ7, and Vβ2, whereas the TCR-α chain is mostly an invariant α chain using the Vα14 and Jα281 segments with a conserved junctional sequence (9, 10). The limited TCR-αβ diversity of NK T cells suggested that these cells interact with a similarly nonpolymorphic ligand (4, 10). Studies using T cell hybridomas derived from NK T cells have clearly identified the nonpolymorphic MHC class Ib CD1 molecule as the ligand recognized by these peculiar TCR (11). NK T cells are remarkable for their ability to produce large amounts of cytokines after TCR stimulation (12), notably IL-4 (12, 13). This prompt IL-4 production by NK T cells has suggested a model in which these cells are one of the major determinants influencing the final TH1/TH2 profile of immune responses (13, 14).

The potential to produce IL-4 and the NK phenotype are two characteristic properties of NK T cells that are likely acquired during their selection by CD1 at an early ontogenic stage (15). This hypothesis has been strengthened by recent studies of Vα14-Jα281 transgenic mice (16), which have increased numbers of NK T cells, increased IL-4 production and augmented baseline levels of IgE and IgG1 (16). Still, a role for the NK-associated molecules during the selection of NK T cells has not been excluded, and a core-
cytomegaly (17). In contrast, TCR-αβ coreceptors as the region that specifically interacts with p56^lck, a tyrosine kinase whose association with the CD4 or CD8 coreceptors is important for optimal T cell activation (18).

A separate question in the development of NKT T cells involves the role of cytokines. The common γ chain (γc), is a critical component of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15. γc-deficient mice have abnormal lymphoid development, with a complete absence of NK T cells, TCR-γδ T cells, and gut-associated intraepithelial lymphocytes (19). In contrast, TCR-αβ T cells and B cells are present, albeit in reduced numbers Therefore, γc^-mice represent a useful system to assess lineage relationships between various lymphoid subpopulations. In this report, we have studied NKT cell development in γc^-mice. The presence of NKT thymocytes in γc^-mice suggest a closer ontogenic relationship of NKT cells to mainstream TCR-αβ T cells than to NKT cells with respect to cytokine dependency. Based on our phenotypic and functional analyses of γc^-NK T thymocytes, we propose and discuss a stepwise model of NKT cell differentiation.

Materials and Methods

Mice. Mice deficient for the common cytokine receptor γ chain, γc (initially identified as the IL-2 receptor γc, reference 19), were maintained in our conventional animal facility and were of a mixed background (129/Ola/BALB/c or 129/Ola/BL/6). For the analysis of NKT-associated markers including NK1.1 and Ly49 expression) were mated to normal BL/6 males or females. Female mice heterozygous for the X-linked γc mutation (from the fourth backcross to BL/6 with confirmed NKT.1 and Ly49 expression) were mated to normal BL/6 males and the subsequent γc^- or γc^- male mice were analyzed for NK1.1 expression. Mice were analyzed between 4 and 10 wk of age.

Cell Preparation and FACS Analysis. Thymocyte and splenocyte (red cell-depleted) suspensions were prepared aseptically in HBSS after pressing through sterile mesh filters. Lymphocytes were isolated using discontinuous Percoll gradients (20) with minor modifications. Cells were stained using combinations of directly conjugated mAbs: FITC-anti-TCR-αβ (clone H57), biotin-anti-heat-stable antigen (HSA, clone J11d), and biotin-anti-Vβ8 (clone F23.1); FITC-anti-IL-7R α chain (21) (purified and locally conjugated by standard methods), PE-anti-HSA, biotin-anti-TCR-αβ, FITC-anti-IL-2R β, FITC-anti-Ly49C, and PE-anti-NK1.1 [all from Pharmingen, San Diego, CA]; PE-anti-CD4, FITC-anti-CD8, and Tricolor-streptavidin (Caltag Laboratories, San Francisco, CA). Three-color immunofluorescence analysis was performed using a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA) and analyzed using CellQuest software.

Quantitative RT-PCR. Total RNA was extracted with acid-guanidium (22) and ethanol-precipitated with the addition of 5 μg of glycogen before resuspension in 20 μl of DEPC water. Reverse transcription and quantitative PCR amplification were carried out as previously described (23) using oligonucleotides specific for Cα, Vα14, and Jα281 (10). It should be stressed that because there is no allelic exclusion for the α chain locus of the TCR (24), only enrichment for a certain VJ combination in a peculiar sample can be detected by PCR analysis using PCR primers specific for Vα and Jα segments. If the amount of starting material is high enough (more than ~2 × 10^4 cell equivalents), in samples that do not contain Vα14-Jα281 invariant α chains (such as those from β2m^-/- mice), there is always a background signal related to the amplification of nonselected out of frame and/or polymorphic TCR-α chains using the same VJ combination. Indeed, polyclonal sequencing of such Vα14-Jα281 PCR products demonstrated their polymorphism (reference 10; data not shown).

In the kinetic PCR method we are using, if one considers two samples containing the same amount of Cα, a shift of n cycles along the x axis of the two amplification curves represents an n-1.8-fold difference in Vα14-Jα281 expression.

In Vivo and In Vitro IL-4 Production. Administration of purified anti-CD3 (clone 145-2C11; 2 μg i.v.) and subsequent in vitro culture of splenocyte suspensions for IL-4 production were performed exactly as described (13). To evaluate cytokine production following stimulation in vitro, CD8^-thymocytes were purified after a one-step killing with anti-CD8 mAb (TiB-211; American Type Culture Collection, Rockville, MD) plus low-toxic M rabbit complement (Cederlane Laboratories, Hornby, Canada). Viable cells were recovered by centrifugation over a density gradient. CD8^-thymocytes (3 × 10^5) were cultured in RPMI-1640 medium, 10% FBS, 50 μM 2-mercaptoethanol, 2 mM glutamine with 3 × 10^4 antigen-presenting cells and soluble anti-CD3 at 5 μg/ml in a total volume of 0.4 ml for 48 h, with or without exogenous cytokines (thymic stromal cell-derived lymphopoietin, TSLP [25], at 10 ng/ml). Supernatants were harvested and IL-4 content measured using the CT.45 cell line. Responses were compared with those elicited by known amounts of murine IL-4.

Results and Discussion

NKT thymocytes develop in the absence of γc^-mice. A semiquantitative PCR approach (23) was used to enumerate NKT cells.

RNA was isolated from thymic tissue, reverse transcribed, and qPCR performed for Cα, Vα14, and Jα281. Figure 1. Vα14-Jα281 invariant α chain is normally expressed in mature thymocytes of γc^-mice. Duplicates of samples of 5 × 10^5 CD8^-thymocytes were obtained from the indicated mice and RNA extracted. After reverse transcription, the indicated genes were amplified and the amount of amplicons quantified at the indicated cycle. Averaged duplicate values are shown. Representative of three independent experiments.

Vα14-Jα281:FIGURE 1. Vα14-Jα281 invariant α chain is normally expressed in mature thymocytes of γc^-mice. Duplicates of samples of 5 × 10^5 CD8^-thymocytes were obtained from the indicated mice and RNA extracted. After reverse transcription, the indicated genes were amplified and the amount of amplicons quantified at the indicated cycle. Averaged duplicate values are shown. Representative of three independent experiments.
by exploiting the fact that these cells exhibit a restricted TCR-α chain repertoire, using the Vα14 segment joined to γc281 (10). We quantitated the amounts of Vα14-γc281 TCR-α chain in CD8- (CD4-CD8- DN) and CD4+ single-positive (SP) thymocytes from γc+, γc-, and βm-/- mice. βm-/- cells were used as control as it has been previously shown that NK T cells require the βm-associated CD1 molecules in order to be selected efficiently (4, 5, 10, 11, 15). As shown in Fig. 1, Cα transcripts were found equally in all three cDNA preparations. The amount of Vα14-γc281 mRNA was similar in both γc+ and γc- thymi (within threefold) and largely increased relative to βm-/- thymi, which lack NK T thymocytes. Direct polyclonal sequencing the Vα14-γc281 amplicons from γc+ and γc- thymi verified the presence of the canonical CDR 3 motif, whereas βm-/- amplicons were polymorphic (data not shown).

The presence of γc- NK T thymocytes was confirmed by analysis of DN thymocytes for the expression of Vβ (Fig. 2). The TCR-β repertoire of DN NK T cells is highly restricted, with ~50% of cells using Vβ8 (4, 7, 10). DN thymocytes from both γc+ and γc- mice contained a population of Vβ8+ cells (~5%), which was not detected in DN thymocytes from βm-/- mice. Percentages of Vβ8+ cells amongst mature (HSAlo) DN thymocytes were also comparable between γc+ (30.3 ± 7.8%) and γc- (20.7 ± 4.7%) mice (data not shown). Taken together, these results suggest that NK T cells are found at the same relative frequency in γc- thymi as in γc+ thymi, although their absolute numbers are reduced 20-fold in parallel with the overall decrease in thymopoiesis seen in γc- mice (19).

These results suggest that generation of NK T cells after interactions with CD1 molecules can proceed in the absence of γc. In this way, selection of NK T cells parallels that of thymocytes containing a subpopulation of TCR-βm+ cells expressing the NK1.1 marker (Fig. 3). In γc- mice, a fraction of the NK1.1+ thymocytes coexpressed Ly49C or Ly49A, and all cells were positive for the IL-2R β and IL-7R α chains (Fig. 3; data not shown). In contrast, no NK1.1+ or Ly49C+ cells were found in thymi from BL/6 γc- mice, although TCR-αβm+ cells were clearly detectable (Fig. 3). In γc- mice, these TCR-αβm+ cells expressed high levels of IL-7R α (like their γc+ counterparts) and somewhat reduced levels of IL-2R β (Fig. 3).

These results, together with the Vα14-γc281-specific PCR data and expression of Vβ8 on DN thymocytes, demonstrate that NK T cells are present in the thymus of γc- mice, although they do not express the NK-β-associated markers. This suggests that the generation and selection of NK T cells can be dissociated from the acquisition of the NK-β-P1 and Ly49 markers, and that the NK phenotype is a contingent phenomenon, which alone cannot be used to define a particular lineage. Concerning the potential function of NK-β-P1 molecules as co-receptors for the recognition of CD1 during selection of NK T cells (2), our results show that NK-β-P1 expression is not strictly required for positive selection of NK T cells on CD1, although we cannot rule out that additional interactions are afforded to the selection process by NK-β-P1 molecules.

The coexpression of Ly49 family molecules on γc- NK T thymocytes is consistent with the NK-associated markers being encoded by their genetically linked loci or the NK gene complex (27), the regulation of which appears to ensure the simultaneous expression of negative (Ly49) and positive (NK-β-P1) signaling molecules on NK cells and NK T cells.
NK thymocytes were isolated and stimulated as described in Materials and Methods. Mice received 2 μg of anti-CD3 intravenously and splenocytes were prepared as described (13). IL-4 bioactivity was assayed using the CT.4S indicator line.

Because NK T cells are selected to a similar degree in the thymus of γc− mice as in γc+ mice, and in the absence of NK K-associated markers, the major determinant of NK T cell selection remains the invariant Vα14-Jα281 TCR-α chain paired with the restricted TCR-β chains (10, 16). Although we cannot rule out a lower avidity reaction in the absence of NK R-P1 or Ly49, we would suggest that the expression of NK K-associated markers is probably the result of additional maturation events after selection, rather than being required for the selection process itself. This also argues against the hypothesis that would make of the NK T cells a peculiar lineage with a correlated expression of the NK K markers together with the Vα14-Jα281 invariant α chain. Indeed, the invariant α chain appears selected at the protein level rather than being produced through a genetic program that selectively recombines Vα14 and Jα281 (10).

Production of IL-4 by γc− NK T thymocytes. The unique ability of NK T cells to produce IL-4 after TCR triggering has been one main characteristic of this lymphoid subset (1, 13, 14). When CD8− thymocytes from γc+ or γc− mice were cultured in vitro with soluble CD3 and antigen-presenting cells, IL-4 release could be detected in the supernatants from γc+ and γc− cells (Table 1), although IL-4 production from γc− cells was relatively weak. We hypothesized that one reason for the low IL-4 production from γc− thymocytes might relate to poor cell viability during the culture period (48 h). We have recently identified a novel cytokine, TSLP, which shares many functional similarities to IL-7 (25), and uses the IL-7R α chain, but not the γc chain for signaling, which can maintain γc+ and γc− thymocytes in vitro (Park et al., unpublished data). As both γc+ and γc− NK thymocytes expressed the IL-7Rα (Fig. 3), we added TSLP to maintain thymocytes during the in vitro assay of IL-4 production. Exogenous TSLP substantially increased the amount of IL-4 produced from γc− NK thymocytes, approximating the levels produced by γc+ cells under these conditions (Table 1). Addition of TSLP to control thymocyte cultures from β2m−/− mice did not result in the generation of IL-4. Therefore, TSLP can effectively substitute for IL-7 in stimulating NK thymocytes in vitro (28). These results are in accord with the recent observations in IL-7 knockout mice, in which NK thymocytes develop but demonstrate a functional defect in IL-4 production after CD3 stimulation (29). Exogenous IL-7 was able to restore the IL-4 response in vitro (29). NK thymocytes from γc− mice also manifest abnormal IL-4 production in vitro in the absence of IL-7Rα engagement; however, this can be restored with TSLP (Table 1).

The property of IL-4 production by NK T cells is likely related to the selection by CD1 at a particular early ontogenetic stage through the invariant Vα14-Jα281 chain paired with Vβ2, Vβ7, or Vβ8. This concept is supported by recent observations using Vα14-Jα281 transgenic mice, which demonstrate an increased frequency of IL-4−producing NK T cells resulting in increased basal levels of serum IgG1 and IgE (16). Our results are consistent with the idea that positive selection of the Vα14-Jα281-bearing TCRs confers the IL-4-producing phenotype. Furthermore, we demonstrate that this unique ability of NK T cells to secrete IL-4 is not dependent on the expression (and therefore function) of the NK K-related molecules.

Absence of NK T cells in the periphery of γc− Mice. Next, we examined whether γc− NK thymocytes, despite their phenotypic abnormalities, would be able to attain their preferential localizations in the periphery. NK T cells nor-

### Table 1. IL-4 Production from NK T Cells: Production of IL-4 from In Vitro–stimulated Thymocytes

| Experiment | Cells | CD3 + APC | CD3 + APC + TSLP |
|------------|------|-----------|------------------|
| 1          | None | 0         | 0                |
|            | CD8− γc+ | 70        | 120              |
|            | CD8− γc− | 20        | 110              |
| 2          | CD8− β2m−/− | <5        | 20               |
|            | CD8− γc+ | 150       | 300              |
|            | CD8− γc− | 20        | 150              |
| 3          | CD8− β2m−/− | ND       | 4                |
|            | CD8− γc+ | ND        | 180              |
|            | CD8− γc− | ND        | 180              |

NK thymocytes were isolated and stimulated as described in Materials and Methods. Mice received 2 μg of anti-CD3 intravenously and splenocytes were prepared as described (13). IL-4 bioactivity was assayed using the CT.4S indicator line.
mally comprise a small percentage of the lymphocytes present in the spleen and lymph nodes (1–3); however, these cells are abundant in the liver (20, 30). To quantitate peripheral NK T cells, lymphocytes from γC+ γC- or β2m-/- mice were isolated from the liver and spleen and the amount of Vα14-Jα281 mRNA was determined. NK T cells were clearly present in the liver and spleens of γC+ mice (Fig. 4; data not shown) as evidenced by the presence of Vα14-Jα281 mRNA. In contrast, levels of Vα14-Jα281 mRNA from γC- liver and spleen preparations were at or below that of β2m-/- mice (which lack NK T cells) and well below that of γC+ controls (at least 200-fold less). Polycyclonic sequencing of Vα14-Jα281 amplicons from γC+, γC-, or β2m-/- samples showed an invariant sequence only in the γC+ samples (data not shown). Flow cytometric analyses confirmed the presence of NKT1.1+ TCR-αβint cells in in-vitro lymphocytes from γC+ mice, which were not detected in preparations from γC- mice (Fig. 5). Lastly, in-vivo administration of anti-CD3 antibodies stimulated IL-4 release from cultured γC+ splenocytes, whereas no IL-4 production could be detected in splenocyte cultures from γC- mice (Table 2). Taken together, these results demonstrate an absence of NKT cells in the liver and spleen of γC- mice.

Our results suggest that one or a combination of IL-2, IL-4, IL-7, IL-9, or IL-15 is necessary for intrathymic maturation and the export/survival of the NK T cells to the peripheral lymphoid organs. The coexpression of IL-7Rα and IL-2Rβ chains on NK thymocytes suggest that IL-2, IL-7, and/or IL-15 may be important in the final differentiation of these cells. Although IL-2-deficient mice have reduced numbers of NK cells (31), NK thymocytes are present in IL-2-/- mice and have normal expression of the NK R-P1 and IL-2Rβ (Bendelac, A., personal communication). Moreover, IL-7-deficient mice display normal percentage of thymic and splenic NK T cells with a normal phenotype (29), and we have not detected a decrease in Vα14-Jα281 transcripts in the thymus, spleen, and liver of IL-2-/-, IL-4-/-, or IL-7-/- mice compared with wild-type controls (Lantz, O., and J.P. DiSanto, unpublished data). Taken together, these results fail to demonstrate the essential role of either IL-2, IL-4, or IL-7 in the final maturation and export of NK thymocytes. However, functional cytokine redundancy (the use of IL-15 in the absence of IL-2, or TSLP in the absence of IL-7) may allow these processes to occur. Further studies using IL-2Rβ-deficient mice (which can be considered as deficient in IL-2 and IL-15) (32) and IL-7Rα-deficient mice (which inactivate IL-7 and TSLP) (33) should help to elucidate the γC-dependent interactions required for induction of NKT-P1 and Ly49 antigens on NK T cells and their export into the periphery.

Table 2. IL-4 Production from NKT Cells: Production of IL-4 after CD3 Injection In Vivo

| Experiment | Mouse | IL-4 from cultured splenocytes |
|------------|-------|-------------------------------|
| 1          | γC+ no.1 | 72                           |
|            | γC+ no.2 | 64                           |
|            | γC- no.1 | <5                           |
| 2          | γC+ no.1 | 62                           |
|            | γC+ no.2 | 50                           |
|            | γC- no.1 | <5                           |
|            | γC- no.2 | <5                           |
the absence of peripheral NK T cells in γc− mice is related to (a) the absence of the NK markers, which would prevent their export to the periphery, (b) incomplete maturation not related to the NK phenotype, or (c) to their nonsurvival in the periphery due to their inability to respond to γc-dependent lymphokines. Furthermore, the precise molecular mechanisms that allow a TCR-mediated signal to induce the acquisition of the NK markers or the ability to produce IL-4 only at a peculiar ontogenic stage remain to be defined.

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Address correspondence to Dr. James P. DiSanto, INSERM U 429, Hôpital Necker, Pavillon Kirmisson, 149 rue de Sèvres, 75743 Paris, France.

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