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Cutting Edge: Thymic NK Cells Develop Independently from T Cell Precursors

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Although NK cells in the mouse are thought to develop in the bone marrow, a small population of NK cells in the thymus has been shown to derive from a GATA3-dependent pathway. Characteristically, thymic NK cells express CD127 and few Ly49 molecules and lack CD11b. Because these NK cells develop in the thymus, the question of their relationship to the T cell lineage has been raised. Using several different mouse models, we find that unlike T cells, thymic NK cells are not the progeny of Rore-expressing progenitors and do not express Rag2 or rearrange the TCRγ locus. We further demonstrate that thymic NK cells develop independently of the Notch signaling pathway, supporting the idea that thymic NK cells represent bona fide NK cells that can develop independently of all T cell precursors. The Journal of Immunology, 2010, 185: 4993–4997.

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t is now recognized that the peripheral NK cell compartment harbors diverse subsets of mature NK cells consistent with specialized functions (1). The origin behind this NK cell diversity remains unclear but might involve microenvironmental cues influencing the terminal differentiation of NK cells in peripheral tissues as well as local developmental pathways that generate distinct NK cell subsets. Whereas NK cell development primarily occurs in the bone marrow (BM) with mature NK cells subsequently seeding peripheral niches (1), we have recently identified a local GATA3-dependent pathway of mouse NK cell development in the thymus generating NK cells with a distinct phenotype (CD127⁺/CD11b⁻/Ly49ph) and functional potential [higher cytokine secretion, lower cytotoxic potential (2)]. Moreover, thymic NK cells are exported to the lymph nodes, where they represent ∼20% of the resident NK cell population (2).

Although the thymic environment harbors other non-T cell lineage cells (including hematopoietic precursors, B cells, and myeloid cells), the presence of a pathway of NK cell development in the thymus evokes the question of their relationship to T cells and/or T cell progenitors. For example, one study proposed that thymic NK cells actually represent NK-like γδ T cells (3), although this is inconsistent with the fact that thymic NK cells develop independently of Rag2 (2). The Takei laboratory reported TCRγ rearrangements in a large fraction of NK cells in the thymus and lymph nodes (4, 5) and suggested that thymic NK cells might share a precursor stage with T cells and thus represent failed T cell precursors (4). In addition, recent data showed that the population of NK cell progenitor cells in the BM encompasses cells with not only NK potential but also T as well as NKT/bipotent precursor cells (6). The relationship of thymic NK cells to classical NK cells, innate T lymphocytes (γδ T cells and NKT cells), and mainstream T cells remains unclear.

Environmental cues coordinate with specific transcription factors to orchestrate lymphocyte development. Essential cytokines for NK development (including thymic NK cells) include IL-15, whereas IL-7 is required for T cell development and thymic NK cells but not for BM and spleen NK cells (2, 7, 8). Concerning transcription factors, the Id2 repressor is required for NK cell development, but not for T cells, whereas Gata3 is necessary for T and thymic NK cells but impacts less on BM/spleen NK cell development (2, 9–11). Thus, the developmental requirements for thymic NK cells do not cleanly dissociate with either classical NK cells or T cells. Concerning the latter, critical signals are delivered by Notch1 that help specify the T cell fate and are reinforced by signals through the retinoic acid-related orphan receptor γ (encoded by Rore), following expression of the pre-TCR in committed pre-T cells (12). In this study, we assess the impact of these critical T cell pathways on thymic NK cell development to
clarify the relationship of these innate cells to T cell precursors and their progeny.

Materials and Methods

Mice

C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA). Rag2-GFP bacterial artificial chromosome (BAC)-transgenic, Mx-cre transgenic Rbpj
t, and Mx-cre transgenic Notch1
t, Rorc(t)-CreTG, ROSA-yellow fluorescent protein (YFP), CD3ε
t, andTCRB
t mice have been described previously (13–18). Mice were analyzed at 6–12 wk of age. All experiments followed institutional guidelines (Animal Care and Use Committee of the Institut Pasteur, Paris, France) and were performed in accordance with French law or with the authorization and approval of the review board of the Veterinary Service from Canton de Vaud (Lausanne, Switzerland).

Flow cytometry and cell sorting

Single-cell suspensions were prepared and stained for intracellular and cell surface proteins as described (2). Abs to Notch1 (22E5.5) and Notch2 (2) were used on FACSDiva software 6.1, BD Biosciences, San Jose, CA) and analyzed using Flowjo software (Tree Star, Ashland, OR). Cells were sorted on an FACSAria II cell sorter (BD Biosciences). Dead cells were excluded using Live/Death fixable Aqua cell stain (Invitrogen, Carlsbad, CA).

PCR assay

Single NK cells were sorted from CD3ε−/−/− mice (thymus; CD127+ cells; spleen; CD127− cells) and γδT cells from the thymus of TCRg2−/− mice as controls. Single-cell PCRs to detect the Vγ2-Jγ2 and Vγ4-Jγ1 rearrangements (according to the Heilig and Tonegawa nomenclature; see Ref. 20) were performed as described (21).

BM chimeras

MX-cre transgenic Rbpj
t mice and Mx-cre transgenic Notch1
t littermates (both CD45.2) were injected five times at 2-d intervals with 150 μg polyinosinic-polycytidylic acid (Sigma-Aldrich, St. Louis, MO). BM cells where the deletion of the corresponding floxed alleles were verified as described (14, 15) (Supplemental Fig. 1) were mixed with wild-type BM (CD45.1) at a 1:1 ratio and injected i.v. into lethally irradiated C57BL/6 mice (CD45.1) to generate Rbpj- or Notch1-deficient BM chimeras. MX-cre-negative Rbpj
t or Notch1
t littermates were treated in the same way to generate control BM chimeras. Mice were analyzed 12 wk postgraft.

Results and Discussion

Most thymic NK cells do not derive from Rorc-expressing precursors and do not express intracellular CD3ε

The transcription factor Rorc is expressed by all developing CD4−CD8− double-positive thymocytes (22). To identify whether thymic NK cells (identified as either CD3ε+ NKp46+ or CD3−NK1.1+ cells) derive from Rorc-expressing committed T cell precursors, we used an in vivo cell fate-mapping approach (16). BAC-transgenic mice expressing the Cre recombinase under the control of the Rorc regulatory elements [Rorc(t)-CreTG mice] were crossed to mice in which the expression of a fluorescence reporter gene (YFP) inserted into the endogenous ROSA26 locus is prevented by a loxp-flanked transcriptional stop cassette [Rosa-YFP mice (16)]. Cre-mediated excision of the stop cassette genetically marks all cells expressing Rorc as well as their progeny with YFP expression (16). Using this system, we found that <8% of NK cells in the thymus of adult mice were progeny of Rorc-expressing progenitors (Fig. 1A, Supplemental Fig. 2), indicating that the vast majority of these cells do not derive from double-positive cells. Moreover, only a small percentage (<3%) of thymic NK cells expressed intracellular CD3ε (Fig. 1B). These data are inconsistent with the idea that thymic NK cells represent masquerading TCRαβ cells (3).

Thymic NK cells do not express Rag2 and do not rearrange the TCRγ locus

It was previously reported that a large proportion of CD127+ and CD127− NK cells from thymus and lymph nodes carry TCRγ rearrangements (4, 5), suggesting that they are derived from CD4−CD8− double-negative (DN) T cell progenitors and might be the product of abortive early T cell development (4). DN T cell precursors can be subdivided into four subsets (DN1–4) based on their differential expression of CD44 and CD25 (Fig. 2A) (23). TCR rearrangements of the β-, γ-, and δ-chains occur at the DN2 and DN3 stages (24). As these rearrangements depend on the presence of Rag1 and Rag2, we used BAC-transgenic mice expressing GFP under the Rag2 promoter (13) to assess Rag2 expression in early DN thymocytes and thymic NK cells. Whereas 11% of DN1 cells, 80% of DN2, and all DN3 cells expressed high levels of GFP (Fig. 2B), <1% of thymic NK cells were GFP+ (Fig. 2B). Moreover, the level of GFP expression by thymic NK cells was considerably lower compared with GFP+ DN1 and DN2 cells (Fig. 2B). These data indicate that essentially all thymic NK cells are not actively rearranging their Ag receptor loci; however, it cannot be excluded that thymic NK cells might derive from Rag-expressing progenitors that have extinguished Rag expression.

Previous studies found at least 50% of thymic NK cells carried TCRγ rearrangements (4, 5), and these authors concluded that thymic NK cells derive from early T cell precursors that had undergone TCRγ rearrangements. However, those analyses were made using in vitro-expanded NK cell cultures isolated from thymus, lymph nodes or spleen (4, 5), and the possibility of a small number of contaminating mature T cells was not rigorously excluded. Moreover, when

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**FIGURE 1.** Rorc expression fate mapping and intracellular CD3ε expression by T cells and thymic NK cells. A. Expression of YFP by CD3ε−NKp46+ (black line) and CD3ε−NKp46− thymocytes (shaded gray) from Rorc(t)-CreTG ROSA-YFP mice. B. Viable thymocytes from adult C57BL/6 mice were stained with the indicated Abs (left panel). Expression of intracellular CD3ε versus NK1.1 (right panel) on gated cells as indicated on the left. Percentages indicate the frequencies of the gated cells.
using freshly isolated splenic NK cell from B6 or in vitro-expanded splenic NK cells from TCR β-/-δ-/- double-deficient mice, only very few NK cells (≈1%) were found to have TCR γ rearrangements (4, 5). We therefore sorted single Lin- NK1.1+CD127+ thymic NK cells from CD3 «/-/ mice (to avoid mature T cell contamination) and directly performed single-cell PCR to detect Vγ2-Jγ2 and/or Vγ4-Jγ1 rearrangements, as these gene segments have been demonstrated to undergo the highest rate of rearrangement (25). Importantly, early T cell precursors from CD3 «/-/ mice have been shown to undergo normal TCR rearrangements (17). We found 1 out of 159 thymic NK cells had both Vγ2-Jγ2 and Vγ4-Jγ1 rearrangements, whereas none of the sorted thymic NK cells carried either only Vγ2-Jγ2 or only Vγ4-Jγ1 rearrangements. This represents a frequency of <1%, which is in agreement with the absence of Rag2 expression by thymic NK cells (Fig. 2). As a control, we found that 30 out of 30 single γδ T cells sorted from TCR β-/- mice and 0 out of 22 sorted splenic CD3ε-/- NK cells from CD3ε-/- mice carried either Vγ2-Jγ2 and/or Vγ4-Jγ1 rearrangements as determined side-by-side in the same single-cell PCR assays. Collectively, these data show that thymic NK cells do not express Rag2 and do not rearrange the TCR γ locus, which is inconsistent with their development from aborted T cell precursors that had previously expressed Rag genes.

**FIGURE 2.** Rag2-GFP expression by DN thymocytes and thymic NK cells. A, CD44 versus CD25 profile on gated CD3-CD4-CD8-Gr-1-CD19- thymocytes from adult Rag2-GFP BAC-transgenic mice. The percentages give the frequencies of the DN subsets (DN1, CD44+CD25+; DN2, CD44+CD25-; DN3, CD44-CD25+; and DN4, CD44-CD25-). B, GFP versus NK1.1 expression as detected in the different DN subsets (as indicated in A).

**FIGURE 3.** Thymic NK cells develop in the absence of Notch signaling. A, Expression of Notch1 (shaded gray) and Notch2 (gray line) by CD3-CD4-CD8-Gr-1-CD19-CD122+NKp46+CD127- thymocytes (left panel) and CD3-CD4-CD8-Gr-1-CD19-CD122-CD25-CD127- splenocytes (right panel) in control and Rbpj-/- BM chimeras 12 wk postreconstitution. Controls are in black (shaded). B, Chimerism among CD3- NKp46+ thymocytes from the different BM chimeras (left panel, control; right panel, Rbpj-deficient) 12 wk postreconstitution is given in percentages. C, CD127 versus CD11b profiles of gated CD45.2+NKp46+CD3- thymocytes from the indicated BM chimeras. Frequency of CD127+ cells is indicated. Results of one representative experiment out of three are shown. D, Absolute numbers (mean and SD) of CD45.2+CD127+ thymic NK cells in controls (littermate, n = 3) and Rbpj-/- BM chimeras (n = 3). p > 0.4.
(Fig. 3A and data not shown). The expression of Notch proteins by splenic CD127+ NK cells was similar to that observed on thymic NK cells (Fig. 3A and data not shown). Thymic NK cells and CD25+ DN thymocytes expressed similar levels of Notch2 at the cell surface, whereas Notch1 was expressed at ~10-fold higher levels by CD25+ DN thymocytes than by thymic NK cells (Fig. 3A). These data suggested that thymic NK cells might derive from a Notch1- and -2–expressing ETP. To assess the role for Notch signaling in thymic NK cell development, we analyzed RBPJ-deficient BM chimeras (14). Among the RBPJ-deficient cells (CD45.2+) in the spleen of RBPJ-deficient BM chimeras, we observed an absence of T cells and marginal zone B cells, as expected (14, 26) (Supplemental Fig. 3A, 3B). The numbers of splenic RBPJ-deficient and control CD127+CD3−NKp46+ NK cells were comparable in the respective BM chimeras (Rbpj−/−/− NK cells: 1.4 × 10^5 ± 7.5 × 10^4 cells versus controls: 2.2 × 10^5 ± 10^5 NK cells; p = 0.35) and the distribution of donor-derived splenic NK cell subsets, as defined by the differential expression of CD11b versus CD27, was not statistically significantly different between controls and mutants (data not shown). Although thymic cellularity was comparable in both types of BM chimeras (Rbpj−/−: 4.2 × 10^7 ± 3.3 × 10^5 versus controls: 3.5 × 10^7 ± 2.1 × 10^5), CD45.2 cells were clearly reduced in the absence of RBPJ (Rbpj−/− cells: 9.8 × 10^5 ± 2.6 × 10^4 versus controls: 2.1 × 10^6 ± 2.9 × 10^5). Nevertheless, thymic NK cells were present in normal percentages among total thymocytes when compared with control BM chimeras (Fig. 3B), and their phenotype (Fig. 3C) and absolute numbers (Fig. 3D) were unaltered in the absence of RBPJ. We found no statistically significant difference in the frequency of CD127+ cells among gated CD3−NKp46+ thymocytes when comparing donor-derived cells (controls to Rbpj−/−; p > 0.4), endogenous cells (control BM chimeras to Rbpj−/− BM chimeras; p > 0.4), or donor-derived to endogenous cells (control: p > 0.2; Rbpj−/−: p > 0.5). Similar results were obtained using Notch1-deficient BM chimeras (data not shown). Although CD127+ NK cells can be generated from BM NK cell progenitor cells and ETPs in vitro using cocultures with OP9 or OP9/DL1 cells [the latter expressing the Notch-ligand DL1 (6)], our results suggest that Notch signals are not mandatory for thymic NK cell development in vivo. In conclusion, our data show that the Notch pathway dissociates development of thymic NK cells from early T cell precursors in vivo.

In conclusion, our data clearly demonstrate that the vast majority of thymic NK cells do not belong to the T cell lineage. Although thymic NK cells can develop in the absence of signals essential for T cell development, it remains possible that thymic NK cells may derive from thymic seeding of the recently described early bipotent NK/T progenitor present in the BM (6). In contrast, DN2 thymocytes, although exhibiting NK cell potential in different experimental systems (27), appear to represent only a marginal substrate for the development of thymic NK cells, at least under mphysiologic conditions, as the latter can develop in absence of all T cell precursors and show little evidence of Ag-receptor rearrangements.

Collectively, our data indicate that thymic NK cells represent bona fide NK cells and are consistent with the notion that peripheral NK cell diversity is not only a consequence of mature NK cell differentiation within various tissue microenvironments/under the influence of issue-derived factors, but also via the local generation of tissue-resident/specific NK cells.

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Disclosures

The authors have no financial conflicts of interest.

References


Figure 1. Conditional deletion of Rbpj in early hematopoietic precursors. A) Southern blot of whole bone marrow genomic DNA of control $Rbpj^{flox/flox}$ (left) and $Rbpj^{-/-}$ mice (right).
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**Figure 2.** Gating strategies to identify CD3+NKp46- and CD3-NKp46+ thymocytes. Thymocytes were stained with the indicated antibodies. A) For the identification of CD3+NKp46- thymocytes cells were gated on CD19-CD3- cells (left) and then on NKp46-NK1.1- cells (right). B) For the identification of CD3-NKp46+ thymocytes CD4-CD8- cells (left) among CD3-CD19- cells (as shown in the left panel of A) were analyzed for expression of NKp46 and NK1.1 (right). CD3-NKp46+ thymocytes are boxed (right).
**Figure 3.** Lymphocyte populations in the periphery of Rbpj−/− versus control BM-chimeras. A) CD4 versus CD8 profiles of gated CD45.2+ splenocytes from Rbpj−/− BM-chimeras 12 weeks after reconstitution of lethally irradiated CD45.1+ C57Bl/6 recipients with mixed bone marrow from CD45.2+Rbpj−/− mice and CD45.1+ C57Bl/6 mice. Frequency of CD4+ and CD8+ cells is indicated for the littermate control BM chimera (left) and Rbpj-deficient BM chimera (right). B) CD23 versus CD21 profiles of gated CD45.2+NKp46+CD3−CD19+ splenocytes from control BM chimeras (left) and Rbpj−/− BM chimeras (right). Frequency of CD23+CD21+ and CD23−CD21high cells is indicated. The percentages indicate the distribution of the different subsets. Results of one representative experiment out of three are shown.